AD_____
(Leave blank)

Award Number:
W81XWH-10-1-0655

TITLE:
Neuroimmune Interactions, Low-Dose Sarin Inhalation, and Gulf War Syndrome

PRINCIPAL INVESTIGATOR:

Mohan Sopori, Ph.D.

CONTRACTING ORGANIZATION:
Lovelace Biomed and Environ Research Institute, Albuquerque, NM 87108

REPORT DATE:

October 2012

TYPE OF REPORT:
Revised Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

X Approved for public release; distribution unlimited

☐ Distribution limited to U.S. Government agencies only;
report contains proprietary information

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
October 2012	Revised Annual	30 September 2011- 29 September 2012

4. TITLE AND SUBTITLE
Neuroimmune Interactions, Low-Dose Sarin Inhalation, and Gulf War Syndrome

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0655

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Mohan Sopori, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Lovelace Biomed and Environ Research Institute
Albuquerque, NM 87122

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT - After the Gulf War, many returning soldiers developed an unexplained set of symptoms termed the Gulf War Syndrome (GWS). The symptoms included neurological and respiratory problems. Many veterans were exposed repeatedly to organophosphates/carbamates, including pyridostigmine bromide (neostigmine bromide), organophosphate pesticides, and low-doses of nerve gas that inhibit acetylcholine esterase activity causing a surge in acetylcholine levels and possibly a cholinergic shock. Chronic lowdose exposure to cholinergic agents, such as the nerve gas sarin increases the brain levels of proinflammatory cytokines, particularly IL-11β, and high levels of IL-1β may induce GWS-like symptoms. Over the past two years, we have shown that cholinergic agents affect neuroimmune communication and mucus formation in the lung. Briefly: (a) cholinergic agents increase IL-β in the brain and chronic braiexposure to low-doses of IL-1β activates tyrosine kinase activities in the spleen and suppresses the immune system. This is a novel neuroimmune communication pathway (Razani-Boroujerdi et al., 2011, PMID: 21671006; Appendix 1). (b) Cholinergic (nicotinic acetylcholine) receptors control mucus formation in the lung (Gundavarapu et al., 2012, PMID: 22578901; Appendix 2) and in the gut (Gundavarapu et al., very preliminary results). Moreover, cholinergic (nicotinic) receptors also control lung development during embryogenesis (Singh et al., 2012 submitted; Appendix 3). Overall these studies show that cholinergic receptors, particularly nicotinic receptors, play a critical role in health and disease, and chronic exposures to cholinergic agents during the Gulf War could have affected thealth status of veterans.

15. SUBJECT TERMS- Cholinergic agents, neuroimmune communication, airway and gut mucus, immune response, gulf War syndrome

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU		USAMRMC

Table of Contents

INTRODUCTION:

A bidirectional communication exists between the brain and immune system (Blalock, 1994; Sopori et al., 1998). Increasing evidence suggest that neurons are also communicating with cells types other than immune cells such as epithelial cells in the periphery. Indeed many receptors, considered originally specific for the nervous system, are also expressed on non-neuronal cells in the periphery. This is true of the cholinergic receptors such as nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs) that are found on neurons, immune cells, and many other cell types, including lung and gut epithelial cells. The mechanism of communication between these cell types is not clear. Interestingly, non-neuronal cells such as the epithelial cells have been shown to be fully capable of making neurotransmitters such as acetylcholine and GABA and express receptors for neurotransmitters (Proskocil et al., 2004; Fuji et al., 2008; Gundavarapu et al., 2012).

After the Gulf War, many returning soldiers developed an unexplained set of symptoms termed the Gulf War Syndrome (GWS); the symptoms included neurological and respiratory problems (Steele, 2000; Thompson et al., 2004). Many veterans were exposed repeatedly to organophosphates/carbamates, including neostigmine bromide (NB), organophosphate pesticides, and low-doses of nerve gas (Berardocco, 1977; US Department of Defense, 2003) that inhibit acetylcholine esterase (AChE). Chronic low dose exposure to cholinergic agents such as the nerve gas sarin induce increased expression of IL-1β in the brain (Kalra et al., 2002; Langley et al., 2004), and increased presence of proinflammatory cytokines, particularly IL-1β may induce GWS-like symptoms (Ferguson and Cassady, 2001). We had previously observed that chronic low-dose exposure to sarin or other cholinergic compounds suppresses the immune system (Kalra et al., 2002; Langley et al., 2004), and we had preliminary evidence suggesting that chronic exposure to low-dose IL-1β also suppresses the immune system. Therefore, we hypothesized that chronic exposure to AChE inhibitors may increase the expression of IL-1β that in turn produces GWS-like symptoms. We proposed to determine: (a) the mechanism by which sarin and IL-1β suppress the immune system, (b) the role of cholinergic receptors in airway responses, (c) whether exposure to cholinergic agents promotes infections, and (d) test the efficacy of potential therapeutic interventions.

BODY

1. **Delay in starting the animal work**: After the grant was funded, we submitted a detailed animal protocol to our IACUC. Because the project involves complex surgeries and animal handling protocols, it took several months of revisions to the protocol to get it approved by the IACUC. The approved protocol was subsequently sent to The USAMRMC Animal Care and Use Review Office (ACURO) for approval. We received the approval for using the animals under this project in the July 2011. However, this provided us the opportunity to design some cell culture experiments and work on tissues from previously sarin-/IL-1β-/nicotine-exposed animals. Both these “approaches” gave very interesting results and are described below.

2. **Chronic CNS exposure to IL-1β suppresses the immune system**: GW veterans exhibit weaker immune responses and higher levels of proinflammatory cytokines (Ferguson and Cassaday, 2001; Skowera et al., 2004). The mechanism of this response is essentially unknown. Chronic low dose exposure to cholinergic agents including sarin suppresses the immune system and induces the expression of IL-1β in the brain (Kalra et al., 2002; Langley et al., 2004). Similarly, nicotine induces IL-1β expression in the brain and suppresses both inflammatory and Th2 responses. IL-1β has been implicated both in neurotoxicity as well as neuroprotection (Stoll et al., 2002). To ascertain the role of IL-1β in

immunosuppression, low levels of IL-1β were chronically infused in rat brains. Interestingly, at early time points IL-1β promoted pyrogenic and inflammatory responses; however, chronic infusion suppressed both innate and adaptive immune response (Razani-Boroujerdi et al., 2011 – **Appendix 1**). Moreover, the infusion caused activation of tyrosine kinases in the spleen. This represents a novel mechanism f or neuroimmune communication and shows that inflammatory cytokines may modulate the immune system through neuroimmune interactions.

3. **Exposure to the cholinergic agent pyridostigmine bromide (neostigmine bromide) promotes airway mucus formation**: Respiratory ailments such as asthma were seen in significantly higher number of GW veterans during the operation Desert Storm and after return to the USA (Lange et al., 2002; Kelsall et al., 2004). A common response to pulmonary ailments is the production of mucus in the airways, which is observed in many lung diseases such as chronic obstructive pulmonary disease, cystic fibrosis, and asthma (Turner and Jones, 2009). Excessive mucus production may impair lung function through exacerbation of infections and increased airway resistance. We observed that cholinergic agents such as acetylcholine and nicotine promoted mucus formation in the airway epithelial cells. This was blocked by nicotinic but not muscarinic receptor antagonists both *in vitro* and *in vivo*. Moreover, activation of α7-nAChRs was critical for mucus formation and used $GABA_AR\alpha2$ receptor pathway to induce MUC5AC/mucus formation in human bronchial epithelial cells. These studies have been recently published (Gundavarapu et al., 2012 - **Appendix 2**). In addition, the idea to block airway mucus formation by nicotinic receptor antagonists has been submitted for patent. We also found that nicotinic cholinergic receptors are intimately associated with lung development and childhood respiratory diseases and blocking these receptors during gestation corrects the changes in lung development and susceptibility to lung diseases (Singh et al., 2012 Submitted; **Appendix 3**). We also have preliminary data suggesting that nicotinic receptors also control gut mucus response to cholinergic agents, indicating that some inflammatory gut diseases might be controlled by cholinergic receptor antagonists; however, these studies are in very early stages.

KEY RESEARCH ACCOMPLISHMENTS

(a) IL-1β may play a critical role in the perception of inflammation by the CNS and the induction of an immunologic "tolerant" state
(b) The immunosuppressive effects of cholinergic agents might be at least partly mediated through their effects on the brain IL-1β.
(c) Intracerebroventricular application of IL-1β leads to activation of tyrosine kinases in the spleen, suggesting a novel mode of neuroimmune communication.
Points a, b, and c are in Appendix 1 (Razani-Boroujerdi et al., 2011; PMID: 21671006)

(d) Airway epithelial cells express α7, α9, and α10-nicotinic, muscarinic (M2), and $GABA_AR\alpha2$ receptors.
(e) Nicotine, acetylcholine, neostigmine bromide increase mucus formation in the airway epithelial cells that can be block by α7-nAChR antagonist but not by α9/α10-nAChR or muscarinic receptor antagonist. This suggests that α7-nAChRs are critical for airway mucus formation and the biological ligand for these receptors is acetylcholine.
(f) Nicotinic receptor antagonists may have therapeutic potential to treat airway mucus formation.
Points d, e, and f are in Appendix 2 (Gundavarapu et al., 2012, PMID: 22578901)

(g) Nicotinic acetylcholine receptors also control lung development during embryogenesis and activation of these receptors by secondhand smoke causes lung developmental changes and increased risk of lung diseases such as asthma (Singh et al., 2011) and bronchopulmonary dysplasia (**Singh et al., submitted; Appendix 3**).

REPORTABLE OUTCOMES

1. Razani-Boroujerdi, S., R. J. Langley, S. P. Singh, R. Kalra, J. C. Philippides, J. Rir-sima-ah, S. Gundavarapu, N. Mishra and M. L. Sopori. 2011. The role of IL-1β in nicotine-induced immunosuppression and neuroimmune communication. *J. Neuroimmune Pharmacol. 6:585-96, 2011.* ***PMID: 21671006***
2. Gundavarapu, Sravanthi, Julie A. Wilder, Neerad C. Mishra, Jules Rir-sim-ah, Raymond J. Langley, Richard J. Jaramillo, Katherine M. Gott, Shashi P. Singh, Juan Carlos Peña-Philippides, Kevin S. Harrod, Ali Imran Saeed, J. Michael McIntosh, Shilpa Buch, and Mohan L. Sopori. 2012. Role of nicotinic receptors and acetylcholine in mucous cell metaplasia, hyperplasia and airway mucus formation in vitro and in vivo. *J Allergy Clin Immunol. 130:770-780, 2012.* ***PMID: 22578901***
3. Singh, Shashi P; Sravanthi Gundavarapu, Kevin R. Smith, Ali Imran Saeed, Neerad C. Mishra, Edward G. Barrett, Matloob Husain, Hitendra S. Chand, Kevin S. Harrod, Raymond Langley and Mohan L Sopori. 2012. Gestational secondhand cigarette smoke causes bronchopulmonary dysplasia blocked by mecamylamine (submitted to *J. Allergy Clin Immunol).*

Conclusions

1. Cholinergic agents may control the immune and inflammatory responses through neuroimmune communication, and IL-1β may be a major molecular entity through which brain senses inflammation.
2. Mucus plays an important role in number of lung and gut diseases, and airway and gut mucus responses are strongly regulated by α7-nicotinic acetylcholine receptors. In the absence of nicotine, acetylcholine acts as a natural ligand for nicotinic acetylcholine receptors for airway mucus production.
3. Cholinergic receptors are important in embryonic lung development and chronic presence of cigarette smoke during gestation may promote lung diseases in children.

REFERENCES

Berardocco D. 1997. DOD, CIA release Khamisiyah modeling data. *Gulf/News 1-3.*

Ferguson E, and Cassaday HJ, 2001. Theoretical accounts of Gulf War Syndrome: from environmental toxins to psychoneuroimmunology and neurodegeneration. Behav Neurol. 13: 133-47.

Fujii, T., Takada-Takatori, Y. & Kawashima, K. Basic and clinical aspects of non-neuronal acetylcholine: expression of an independent, non-neuronal cholinergic system in lymphocytes and its clinical significance in immunotherapy. *J Pharmacol Sci* **106**, 186-192 (2008).

Gundavarapu, Sravanthi, Julie A. Wilder, Neerad C. Mishra, Jules Rir-sim-ah, Raymond J. Langley, Richard J. Jaramillo, Katherine M. Gott, Shashi P. Singh, Juan Carlos Peña-Philippides, Kevin S. Harrod, Ali Imran Saeed, J. Michael McIntosh, Shilpa Buch, and Mohan L. Sopori. 2012. Role of nicotinic receptors and acetylcholine in mucous cell metaplasia, hyperplasia and airway mucus formation in vitro and in vivo. *J Allergy Clin Immunol. 130:770-780, 2012.*

Kalra R, et al. 2002.Subclinical doses of the nerve gas sarin impair T cell responses through the autonomic nervous system. *Toxicol Appl Pharmacol. 184: 82-87.*

Kelsall HL, Sim MR, Forbes AB, McKenzie DP, Glass DC, Ikin JF, Ittak P, Abramson MJ. Respiratory health status of Australian veterans of the 1991 Gulf War and the effects of exposure to oil fire smoke and dust storms. Thorax 2004, 59:897-903.

Lange JL, Schwartz DA, Doebbeling BN, Heller JM, Thorne PS. Exposures to the Kuwait oil fires and their association with asthma and bronchitis among gulf war veterans. Environ Health Perspect. 2002. 110:1141-6.

Langley R, et al. 2004. Central but not the peripheral action of cholinergic compounds suppresses the immune system. *J Neuroimmunol. 148: 140-145.*

Proskocil, B.J.*, et al.* Acetylcholine is an autocrine or paracrine hormone synthesized and secreted by airway bronchial epithelial cells. *Endocrinology* **145**, 2498-2506 (2004).

Razani-Boroujerdi, S., R. J. Langley, S. P. Singh, R. Kalra, J. C. Philippides, J. Rir-sima-ah, S. Gundavarapu, N. Mishra and M. L. Sopori. 2011. The role of IL-1β in nicotine-induced immunosuppression and neuroimmune communication. *J. Neuroimmune Pharmacol. 6:585-96, 2011.*

Singh, Shashi P; Sravanthi Gundavarapu, Kevin R. Smith, Ali Imran Saeed, Neerad C. Mishra, Edward G. Barrett, Matloob Husain, Hitendra S. Chand, Kevin S. Harrod, Raymond Langley and Mohan L Sopori. 2012. Gestational secondhand cigarette smoke causes bronchopulmonary dysplasia blocked by mecamylamine (submitted to *J. Allergy Clin Immunol).*

Skowera A, et al. 2004. Cellular immune activation in Gulf War veterans. *J Clin Immunol. 24: 66-73.*

Steele L. 2000. Prevalnce and patterns of Gulf War illness in Kansas veterans: association of symptoms with characteristics of person, place, and time of military service. *Am J Epidemiol. 152: 992-1002.*

Stoll G, Jander S, Schroeter M (2002) Detrimental and beneficial effects of injury-induced inflammation and cytokine expression in the nervous system. Adv Exp Med Biol 513:87–113.

Thompson et al. 2004. The benefits and risks associated with cholinesterase inhibitor therapy in Alzheimer's disease. *Expert Opin Drug Saf.3:425-440.*

Turner J, Jones CE. Regulation of mucin expression in respiratory diseases. Biochem Soc Trans 2009; 37:877-81.

US Dept Defense (2003). Environmental Report. Pesticides. *www.gao.gov/htext/d04159.html*.

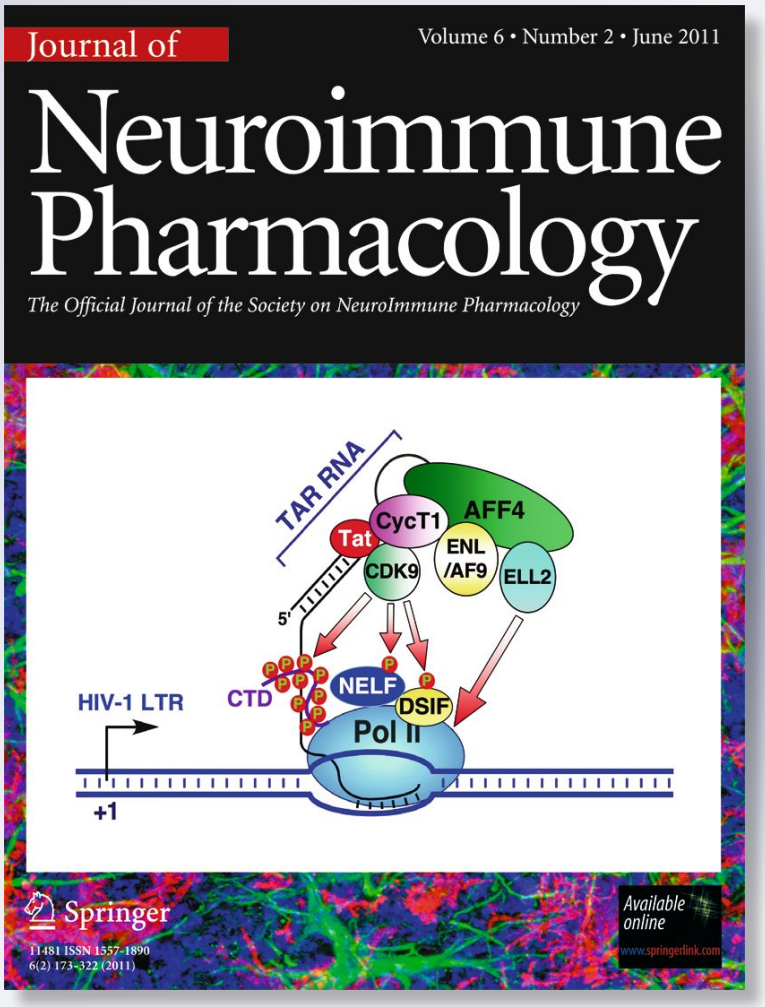
The Role of IL-1# in Nicotine-Induced Immunosuppression and Neuroimmune Communication

Seddigheh Razani-Boroujerdi, Raymond J. Langley, Shashi P. Singh, Juan Carlos Pena-Philippides, Jules Rir-sima-ah, Sravanthi Gundavarapu, et al.

Journal of Neuroimmune Pharmacology

ISSN 1557-1890

J Neuroimmune Pharmacol
DOI 10.1007/
s11481-011-9284-5



Springer

Your article is protected by copyright and all rights are held exclusively by Springer Science+Business Media, LLC. This e-offprint is for personal use only and shall not be self-archived in electronic repositories. If you wish to self-archive your work, please use the accepted author's version for posting to your own website or your institution's repository. You may further deposit the accepted author's version on a funder's repository at a funder's request, provided it is not made publicly available until 12 months after publication.

Author's personal copy

J Neuroimmune Pharmacol
DOI 10.1007/s11481-011-9284-5

ORIGINAL ARTICLE

The Role of IL-1β in Nicotine-Induced Immunosuppression and Neuroimmune Communication

Seddigheh Razani-Boroujerdi · Raymond J. Langley · Shashi P. Singh · Juan Carlos Pena-Philippides · Jules Rir-sima-ah · Sravanthi Gundavarapu · Neerad C. Mishra · Mohan L. Sopori

Received: 9 December 2010 / Accepted: 27 May 2011
© Springer Science+Business Media, LLC 2011

Abstract Although a number of inflammatory cytokines are increased during sepsis, the clinical trials aimed at down-regulating these mediators have not improved the outcome. These paradoxical results are attributed to loss of the "tolerance" phase that normally follows the proinflammatory response. Chronic nicotine (NT) suppresses both adaptive and innate immune responses, and the effects are partly mediated by the nicotinic acetylcholine receptors in the brain; however, the mechanism of neuroimmune communication is not clear. Here, we present evidence that, in rats and mice, NT initially increases IL-1β in the brain, but the expression is downregulated within 1–2 week of chronic exposure, and the animals become resistant to proinflammatory/pyrogenic stimuli. To examine the relationship between NT, IL-1β, and immunosuppression, we hypothesized that NT induces IL-1β in the brain, and its constant presence produces immunological "tolerance". Indeed, unlike wild-type C57BL/6 mice, chronic NT failed to induce immunosuppression or downregulation of IL-1β expression in IL-1β-receptor knockout mice. Moreover, while acute intracerebroventricular administration of IL-1β in Lewis (LEW) rats activated Fyn and protein tyrosine kinase activities in the spleen, chronic administration of low levels of IL-1β progressively diminished the pyrogenic and T cell proliferative responses of treated animals. Thus, IL-1β may play a critical role in the perception of inflammation by the CNS and the induction of an immunologic "tolerant" state. Moreover, the immunosuppressive effects of NT might be at least partly mediated through its effects on the brain IL-1β. This represents a novel mechanism for neuroimmune communication.

Keywords Nicotine · IL-1β · Immunosuppression · Neuroimmune communication · Fyn

Abbreviations

$[Ca^{2+}]_i$	intercellular calcium concentration
NT	nicotine
PTK	protein tyrosine kinase
HPA	hypothalamus-pituitary-adrenal
LEW	Lewis
KO	knockout
WT	wild-type
ICV	intracerebroventricular
aCSF	artificial cerebrospinal fluid
AFC	antibody-forming cell
qPCR	real-time PCR
CORT	corticosterone

Introduction

Cigarette smoking is a major health risk factor and contributes to over three million premature deaths annually worldwide. Many adverse health effects of cigarette smoke might stem from its immunosuppressive effects (Holt and Keast 1977; Sopori 2002). To that end, we and others have

This work was supported in part by grants from the US Army Medical Research and Material Command (GW093005), the National Institutes of Health (RO1 DA017003), and the Defense Threat Reduction Agency (HDTRA 1-08-C-002)

S. Razani-Boroujerdi and R. Langley contributed equally to the work.

S. Razani-Boroujerdi · R. J. Langley · S. P. Singh · J. C. Pena-Philippides · J. Rir-sima-ah · S. Gundavarapu · N. C. Mishra · M. L. Sopori (✉)
Respiratory Immunology Program,
Lovelace Respiratory Research Institute,
2425 Ridgecrest Dr., S.E.,
Albuquerque, NM 87108, USA
e-mail: msopori@lrri.org

Published online: 14 June 2011

Author's personal copy

shown that chronic inhalation of cigarette smoke suppresses the immune system in humans and experimental animals; reviewed in (Sopori et al. 1998a; Stampfli and Anderson 2009). Nicotine (NT), the major immunosuppressive compound in cigarette smoke, may affect immune and inflammatory responses through the central and peripheral mechanisms (Sopori et al. 1998b; Sopori 2002; Wang et al. 2003; van Westerloo et al. 2005; Mishra et al. 2008). We have reported that while acute NT increases intracellular calcium $[Ca^{2+}]_i$ in T cells (Razani-Boroujerdi et al. 2007), chronic exposure of animals to NT causes T cell anergy through constitutive activation of protein tyrosine kinase (PTK) activity, including Fyn, production of inositol-1,4,5-trisphosphate, and depletion of inositol-1,4,5-trisphosphate-sensitive Ca^{2+} stores, leading to loss of TCR-mediated elevation in $[Ca^{2+}]_i$ (Geng et al. 1996; Kalra et al. 2000). The immunosuppressive effects of chronic NT treatment in vivo are, at least partially mediated through the nicotinic acetylcholine receptors in the CNS, and the effects are independent of the hypothalamus-pituitary-adrenal (HPA) axis (Sopori et al. 1998b; Singh et al. 2000).

The mechanism by which NT modulates the immune/inflammatory system through the CNS is not clear. There is evidence that cytokines in the brain play an important role in the regulation of the inflammatory and anti-inflammatory responses. For example, neuroinflammation may damage neurons, but it also confers neuroprotection (Stoll et al. 2002). Increasing evidence suggests that proinflammatory cytokines such as IL-1β also provide neuroprotection and dampen neuroinflammation (Stoll et al. 2002; Jin et al. 2009; Pinteaux et al. 2009). Similarly, head trauma/injury, usually associated with a pyrogenic response (Badjatia 2009), also leads to systemic inflammatory response syndrome followed by a compensatory anti-inflammatory response syndrome (Lenz et al. 2007; Lu et al. 2009). Proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 have been implicated in acute inflammatory responses (Dinarello 2000); however, there is increasing evidence that early presence of these cytokines might have a protective role in sepsis (Torre et al. 1994; del Rey and Besedovsky 2000; Kox et al. 2000). Similarly, pretreatment with sublethal doses of endotoxin increases proinflammatory cytokine production, but protects against septic peritonitis through a mechanism independent of adaptive immunity (Urbaschek and Urbaschek 1987; Varma et al. 2005); these results can be achieved by pretreatment with IL-1β and TNF-α (Urbaschek and Urbaschek 1987). In this communication, we present evidence that (a) acute NT exposure induces IL-1β in the brain, but the response is lost through continued exposure to NT, leading to an immunologic "tolerant" state, and (b) while an acute administration of IL-1β in the brain activates splenic T cells, continued exposure suppresses the pyrogenic response and T cell function.

Materials and methods

Animals

Male pathogen-free Lewis (LEW) rats were purchased from Harlan Sprague-Dawley Farms (Branchburg, NJ, USA), and IL-1 receptor knockout (IL-1R KO) mice and wild-type (WT) control (C57BL/6) mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Rats and mice were housed individually in class 100 air quality rooms with 12-h light/dark cycle; food (Teklad certified diet) and water were provided ad libitum. Animals were maintained at the thermo-neutral temperatures, i.e., 25±1°C (rats) and 30±1°C (mice). Three- to 4-month old animals were used in these experiments. The Lovelace Respiratory Research Institute IACUC reviewed and approved these studies.

Reagents

Monoclonal antibodies to the rat αβ-TCR and the appropriate isotype control antibodies were purchased from PharMingen (San Jose, CA, USA), and the anti-$p59^{fyn}$ antibody was purchased from Upstate Biotechnology (Lake Placid, NW, USA). Other reagents were obtained from the following vendors: recombinant rat IL-1β (R & D Systems, Inc., Minneapolis, MN, USA), guinea pig complement (Cedarlane Laboratories, Ltd, Burlington. Ontario, Canada), chlorisondamine (Tocris Cookson, Ltd., Ellisville, MO, USA), (±) NT base (MP Biomedicals, Inc., Santa Ana, CA, USA), and P^{32}-ATP (MP Biomedicals). Unless mentioned otherwise, all other reagents, including acetyl-methyl ester of indo-1 and Con A were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

IL-1β treatment

For constant administration, IL-1β (100 ng/kg/day) was given through a subcutaneous (s.c.) implanted Alzet miniosmotic pumps (Alzet Corp., Cupertino, CA, USA) that delivered a volume of 0.25 μl/h. The pumps were implanted as described (Geng et al. 1996). Briefly, rats were anesthetized with isoflurane-oxygen and shaved at the base of the neck. A pocket of approximately 2 cm was made by a transverse incision under the skin, and the pumps were placed in the pocket with the delivery end of the pump facing toward the bottom of the pocket. For intracerebroventricular (ICV) delivery, a 5-mm long, 28-gauge stainless steel cannula was placed stereotaxically into the rat, and connected to a s.c. -implanted mini-osmotic pump (ALZET Brain Infusion Kit, Alzet Corp) as described (Sopori et al. 1998b). Rats were anesthetized

Author's personal copy

with a mixture of ketamine (85 mg/kg) and xylazine (15 mg/kg). For ICV placement of cannulas, the coordinates were 1 mm posterior to the bregma and 1.4 mm lateral to the midline. Miniosmotic pumps were filled with IL-1β in artificial cerebrospinal fluid (aCSF) or aCSF only (control). In some rats, a single injection of 50 ng of IL-1β was given either by a s.c. injection in 0.1 ml saline or administered in 6 μl of aCSF in ICV cannulas placed surgically 1 week prior to IL-1β injection.

NT treatment

Nicotine was administered as (±) NT base through s.c.-implanted miniosmotic pumps that delivered 2 mg of the NT/day/kg body wt in C57BL/6 mice and LEW rats. In rats, this amount of NT raises the blood cotinine (the main metabolic byproduct of nicotine) level equivalent to humans smoking ≤1 packet of cigarettes/day (Geng et al. 1995). To study acute effects of NT, some mice received a single intraperitoneal (i.p.) injection of 7.5 μg of NT, while LEW rats received a single i.p. injection of 62.5 μg of NT.

Immunizations

To determine the antibody-forming cell (AFC) response, 4 days prior to sacrifice, animals were injected with 5×10^8 sheep red blood cell (SRBC) intravenous (i.v.) and i.p. into rats and mice, respectively (Sopori et al. 1989).

Collection of tissues and purification of T cells

Rats were scarified by isoflurane inhalation; some animals were perfused through the heart with endotoxin-free saline prior to the removal of the brain. Brains were collected, dissected longitudinally into halves, and placed in tubes containing TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA), snap frozen in liquid nitrogen, and stored at −80°C for later RNA extraction. Spleens were harvested and cell suspensions made as described (Razani-Boroujerdi et al. 1994a). Briefly, spleens were pressed through stainless steel mesh and treated with buffered NH_4Cl solution to lyse red blood cells. Cells were washed 3 times with PBS and resuspended in complete tissue culture medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, and 1% penicillin/streptomycin). Purified splenic T cells were obtained by MACS separation (Miltenyi Biotec Inc., USA). Spleen cells were suspended in PBS containing 2 mM EDTA and 0.5% BSA, and incubated with Rat Pan T cell Micro beads (Miltenyi Biotec) for 15 min at 4°C, washed with the same buffer, and loaded onto magnetized columns. The column was moved out of the magnetic field, and the retained cells (purified T cells) were eluted from the column with the buffer.

Assay for AFC response

The primary direct AFC response was determined by the Cunningham and Szenberg method as described (Sopori et al. 1989). Briefly, spleen cells were mixed with 2% SRBC and 20 μl of guinea pig complement (pre-absorbed on SRBC) in a final volume of 140 μl. Aliquots were distributed in duplicates into Cunningham slide chambers, incubated for 45 min at 37°C, and counted under low-power microscope. Results were expressed as AFC/10^6 spleen cells.

Assay for T cell proliferation

Proliferative responses were performed as described (Geng et al. 1995). Briefly, in a final volume of 0.2 ml of complete medium, 2×10^5 spleen cells were cultured in triplicate in flat-bottomed, 96-well microtiter plates in the presence and absence of indicated concentrations of Con A or anti-αβ-TCR monoclonal antibody. Unlike the mouse and human T cells, rat T cells are activated to proliferate with anti-TCR antibodies without anti-CD28. Plates were incubated at 37°C in a 5% CO_2 atm. After 48 h, culture wells were labeled with 0.5 μCi of [^{3}H]-thymidine (New England Nuclear Corp, Newton, MA, USA) and harvested 24 h later by a Skatron cell harvester (Molecular Devices Inc, Sunnyvale, CA, USA). Samples were counted in a liquid scintillation counter and results expressed as the mean cpm ± SEM of triplicate cultures.

Assay for intracellular ionized calcium in lymphocytes

The intracellular calcium level ($[Ca^{2+}]_i$) of splenocytes was measured using acetyl-methyl ester of indo-1 under the conditions described (Razani-Boroujerdi et al. 1994a). Briefly, cells (5×10^6/ml) were incubated at 37°C for 30 min with 5 μM acetyl-methyl ester of indo-1 in loading medium (PBS containing, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 3% FCS). After washing, cells were suspended in loading medium and incubated at 37°C for 30 min in 5% CO_2. Cells were kept in the dark on ice until the assay. Before each measurement, 1 ml of the cell suspension was washed and resuspended in 2 ml of the loading medium. The $[Ca^{2+}]_i$ of cells was determined by spectrofluorometry in a PTI Deltascan fluorometer (Photon Technology International, Inc., Birmingham, NJ, USA) at 37°C with constant, gentle stirring. Recording of cell fluorescence was started 60 s before the addition of the αβ anti-TCR and the secondary antibody in rats, and anti-CD3 plus

Author's personal copy

anti-CD28 antibodies in mice. $[Ca^{2+}]_i$ was calculated as previously described (Razani-Boroujerdi et al. 1994b).

Assay for PTK and Fyn activities

Protein tyrosine kinase activity was determined by immunoblotting using anti-phosphotyrosine antibodies (Geng et al. 1996). Briefly, purified spleen cells were suspended in the complete medium and incubated with anti-TCR antibody (2 μg/ml) or an equivalent amount of isotype control antibody for 2 min at 37°C. The reaction was stopped by the addition of ice-cold PBS, and the cell pellet was lysed in cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.25% sodium deoxycholate, 1% Nonidet P-40, 1 mM EDTA, 50 mM NaF, 1 mM activated Na_3VO_4, and protease inhibitors: 1 mM PMSF, and 1 μg/ml each of aprotinin, leupeptin, pepstatin). The lysates were clarified by centrifugation and aliquots of the lysate boiled in Laemmli sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 10 mM DTT, and 0.001% bromophenol blue) for 5 min. Protein concentration was determined by the bicinchoninic acid method (Thermo Fisher Scientific Inc., Rockford, IL, USA), and equal amounts of proteins (10–20 μg) were electrophoresed on 7.5% SDS-PAGE and transferred on polyvinylidene difluoride membranes. The blots were blocked with 5% dry skim milk protein (Upstate Biotech. Inc.) in 10 mM Tris-HCl pH 7.4 containing 150 mM NaCl for 1.5 h at room temperature. The blots were washed and probed with anti-phosphotyrosine monoclonal antibody and developed with HRP-conjugated second antibody. Fyn kinase activity of the lysates was determined essentially by the method of Gould and Hunter (Gould and Hunter 1988). Briefly, 10 μl of anti-$p59^{fyn}$ antibody was added to 500 μl of cell lysate (1 mg protein/ml) and incubated overnight at 4°C on a rocker. Protein-AG (50 μl) was added to the sample, and the mixture was incubated at 4°C for 3 h. The immunoprecipitates were washed 3X with RIPA buffer and 2X with the kinase buffer (50 mM Tris-HCl, pH 7.4, 3 mM $MnCl_2$, 0.1 mM Na_3VO_4), and resuspended in the kinase buffer. The kinase activity of the immunoprecipitate was assayed by incubating the immunoprecipitates with 0.2 μg of acid-treated enolase (Sigma-Aldrich) and γ-^{32}P-ATP (10 μCi) for 20 min at room temperature (Cooper et al. 1984; Gould and Hunter 1988). The reaction was stopped by the addition of Laemmli sample buffer, boiled for 5 min, and the samples analyzed on 10% SDS-PAGE. The gel was dried, and the phosphorylated enolase was visualized by autoradiography.

Real-time PCR (qPCR) for IL-1β mRNA expression

Total RNA was isolated from the frozen brain as described (Razani-Boroujerdi and Sopori 2007). Briefly, tissues were homogenized in the presence of TRI Reagent (Molecular Research Center). Total RNA was isolated using the BCP phase separation reagent (Molecular Research Center Inc.). RNA was precipitated by 2-propanol and washed with 75% ethanol. The RNA pellet was dried for a short time, resuspended in RNase-free water, and quantitated spectrophotometrically. The IL-1β and GAPDH primers and probes were purchased from Applied Biosystem (Foster City, CA). qPCR was performed on the Prism 7900 HT sequence Detection System (Life Technologies Corp., Carlsbad, CA, USA), using the one-step RT-PCR master mix (Life Technologies Corp.), and standard protocols. PCR was performed for 40 cycles with denaturation (95°C for 15 s) and annealing (60°C for 60 s). All results were derived from the linear amplification curve and normalized to GAPDH. The ΔΔCT method was used to calculate the fold change in IL-1β expression.

Assay for corticosterone

Serum corticosterone (CORT) levels were measured using the instructions and reagents provided with a ^{3}H-CORT radioimmunoassay kit (ICN Pharmaceuticals Inc., Costa Mesa, CA, USA)

Measurement of the deep body temperature (Tb)

One week before the implantation of IL-1β delivering miniosmotic pumps, rats were implanted intra-abdominally with biotelemeters (model VM-FH; Mini-Mitter Co., Bend, OR, USA) as reported (Sopori et al. 1998a; Sopori et al. 1998b). After the implantation, the animals were housed individually in plastic cages in special temperature-controlled rooms at 25°C±0.1°C. Signals for Tb were collected at 5-min intervals with a peripheral processor (Dataquest III system) connected to a personal computer. To test the pyrogenic properties of IL-1β contained in the miniosmotic pumps after 14 days of implantation, the contents of the pumps were harvested, pooled, and injected into biotelemeters implanted rats i.v. Tb was recorded following the injection of the contents of miniosmotic pumps or vehicle.

Statistical analysis

Statistical comparisons between three or more experimental groups were performed using a one-way analysis of variance. The Scheffe *post hoc* test was used to determine the significance among groups, and the Student's *t*-test was to compare the means between two groups (CON and IL-1β-treated). These statistical procedures were performed using ABSTAT (Anderson-Bell Corp., Arvada, CO, USA).

Author's personal copy

Results

Acute exposure to NT transitorily increases IL-1β expression in the brain

We and others have shown that NT is anti-inflammatory (Sopori 2002; Scott and Martin 2006; Ulloa and Wang 2007) and reduces the pyrogenic response to turpentine-induced sterile abscess (Sopori et al. 1998a; Sopori et al. 1998b; Razani-Boroujerdi et al. 2004). Because the brain IL-1β is an important cytokine for the pyrogenic response to turpentine (Horai et al. 1998; Kozak et al. 1998), we ascertained whether NT affected the expression of IL-1β in the brain. C57BL/6 mice were treated with a single i.p. injection of 7.5 μg of NT, and the brain IL-1β expression was determined by qPCR analysis at various times after NT injection. Surprisingly, within 1 h after NT treatment, the expression of IL-1β in the brain increased significantly and peaked at around 24 h post-NT administration (Fig. 1A). Similar changes in the brain IL-1β expression were observed when LEW rats were challenged with 62.5 μg of NT via single i.p. injection (Fig. 1B). Chronic treatment of C57BL/6 mice with NT through Alzet miniosmotic pumps increased IL-1β mRNA that peaked at 4 d and returned to the baseline around 8 d (Fig. 1C); a similar increase in IL-1β was also observed after chronic NT treatment of LEW rats that peaked at one wk but reverted to the baseline levels around 3 weeks after treatment (Fig. 1D). Thus, both acute and chronic NT treatments increase the brain expression of IL-1β in both mice and rats; however, even in the continued presence of NT, the cytokine expression dropped within 2 to 3 weeks of chronic NT exposure. Interestingly, intramuscular (i.m.) administration of small quantities of turpentine (a model for sterile abscess) increased IL-1β expression in the brain of naïve control rats; however, animals treated chronically with NT for 2 to 3 weeks, failed to increase IL-1β after the turpentine treatment (Fig. 2). The suppression of cytokine expression by chronic NT-treatment was not absolute, and larger doses of LPS broke the state of unresponsiveness and induced production of proinflammatory cytokines, including IL-1β and TNF-α (not shown). These results suggest that following the early increase in IL-1β, chronic NT induces a state of unresponsiveness ("tolerance") to proinflammatory/pyrogenic stimuli; however, the tolerance is not total and is broken by stronger proinflammatory stimuli.

Unlike WT mice, IL-1R KO mice do not exhibit NT-induced "tolerance"

To ascertain whether the early increase in IL-1β and its interaction with IL-1R were obligatory for the induction of

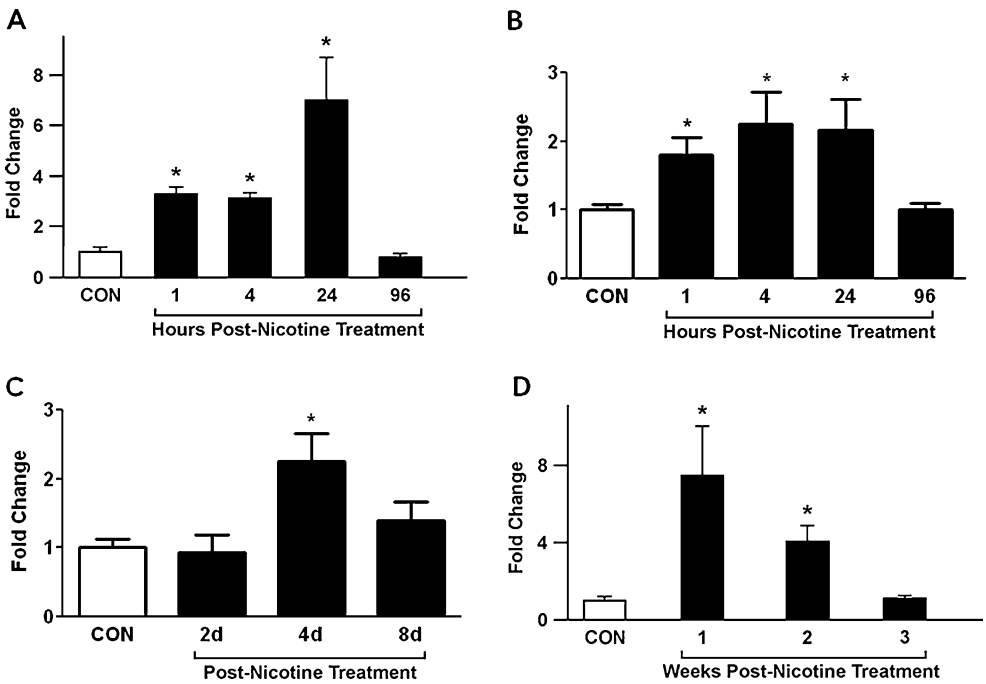


Fig. 1 NT treatment induces 1L-1β expression in the brain. (**A**) C57BL/6 mice were injected with single i.p. injection of 7.5 μg NT and, at various times following NT administration, the brain mRNA was analyzed by qPCR for IL-1β expression. (**B**) Brain IL-1β qPCR analysis of LEW Rats injected with single i.p. injection of 62.5 μg NT at various time points after administration. (**C**) C57BL/6 mice were chronically exposed to NT (2 mg/kg/day) through Alzet miniosmotic pumps, and brain tissues were analyzed by qPCR for 1L-1β expression at 2 days, 4 days and 8 days post NT treatment. (**D**) qPCR analysis of IL-1β expression in the brain of LEW Rats chronically exposed to NT (2 mg/kg/day) 1–3 week post NT treatment. Bar graphs represent mean ± SEM from five rats/group. * $P \leq 0.05$

Author's personal copy

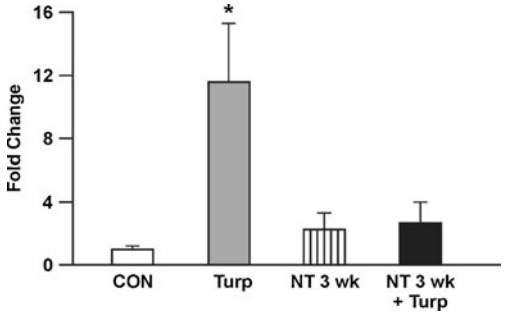


Fig. 2 Chronic NT exposure blocks turpentine-induced expression of IL-1β in brain. Rats were exposed to saline (CON) or NT for 3 weeks and then injected i.m. with turpentine (Turp). At 24 h after turpentine injection, brain mRNA was analyzed for IL-1β expression by qPCR. Results are mean ± SEM from five rats/group. * $P \leq 0.05$

NT-induced "tolerance", we compared the brain IL-1β expression in WT and IL-1R KO C57BL/6 mice after a 3-weeks exposure to NT. Although the IL-1R KO mice did not exhibit as robust NT-induced IL-1β expression as WT control mice, the brain IL-1β expression remained significantly higher than WT mice even after 3-weeks of NT exposure (Fig. 3). Thus, unlike normal mice, where brain IL-1β expression returned to baseline after 1 to 3 weeks of exposure, increased IL-1β-induced expression persisted in IL-1R KO mice. Therefore, it is likely that production of IL-1β and the interaction between IL-1β and IL-1R during the early phase of NT treatment are critical for the induction of cytokine-induced suppression during the chronic phase of NT treatment.

IL-1R KO mice are resistant to the immunomodulatory effects of NT

Nicotine inhibits the T-cell-dependent antibody response, T cell proliferation, and inflammatory responses (Sopori et al. 1989; Sopori et al. 1998a; Wang et al. 2003; Razani-Boroujerdi et al. 2004; Mishra et al. 2008). To examine whether the immunosuppressive effects of NT were related to IL-1R-mediated responses, IL-1R KO and WT mice were chronically exposed to NT for 3 week, and spleen cells were examined for the AFC response to SRBC, Con A-induced T cell proliferation, and anti-TCR/CD28-stimulated rise in $[Ca^{2+}]_i$. Although IL-1R KO mice exhibited a significantly lower anti-SRBC AFC response than WT mice, NT inhibited the AFC (Fig. 4A) and Con A (Fig. 4B) responses in WT but not in IL-1R KO mice. Similarly, NT significantly blunted the anti-TCR/CD28-induced rise in $[Ca^{2+}]_i$ in WT but not IL-1R KO spleen cells (Fig. 5). These data indicate that IL-1R KO mice are resistant to the immunosuppressive effects of NT, and IL-1β may play an important role in mediating the NT-induced immunosuppression.

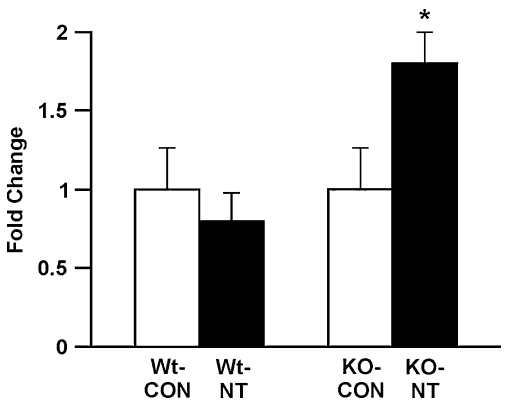


Fig. 3 Chronically NT-treated IL-1R KO mice have higher brain expression of IL-1β mRNA than WT C57BL/6 mice. Mice (IL-1R KO and WT) were treated with NT (1 mg/kg/day) via Alzet miniosmotic pumps, and brain tissues were analyzed for 1L-1β expression by qPCR after 3 weeks of NT treatment. Bar graphs represents mean ± SEM from five mice/group. * $P \leq 0.05$

ICV but not s.c. exposure to IL-1β inhibits the AFC response

To ascertain whether chronic exposure to IL-1β is immunosuppressive, rats were given IL-1β (100 ng/day/Kg) either centrally (ICV) or peripherally (s.c.) via miniosmotic pumps for 1 and 2 week. The animals were

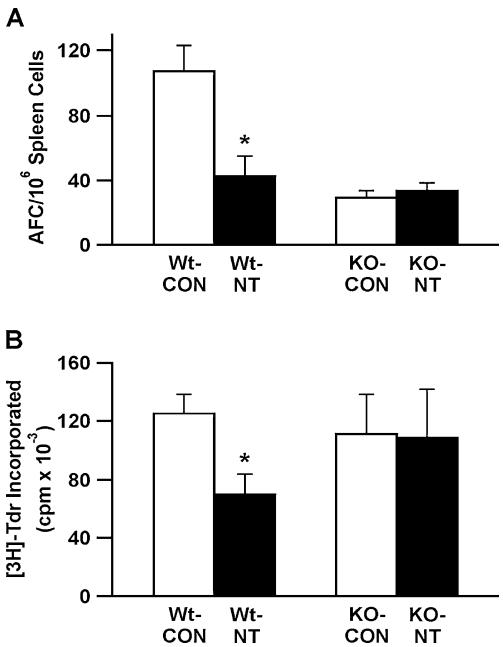


Fig. 4 Chronic NT treatment does not affect the immune responses of IL-1R-KO mice. WT and IL-1R-KO mice (n=6/group) were implanted with NT (2 mg/kg/day)- or PBS-containing Alzet miniosmotic pumps for 3 weeks. Four days prior to sacrifice, animals were immunized with SRBC, and the spleen cells were analyzed: (**A**) AFC/10^6 spleen cells; (**B**) Con A-induced proliferation as described in Materials and methods. Bar graphs represent mean ± SEM. * $P \leq 0.05$

Author's personal copy

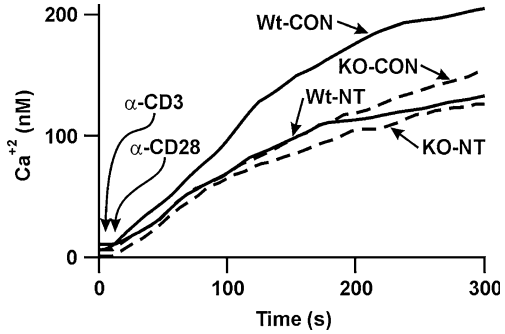


Fig. 5 Chronic NT treatment does not affect the TCR-mediated $[Ca^{2+}]_i$ responses of IL-1R-KO spleen cells. WT and IL-1R-KO mice (n=6) were treated with NT or PBS (CON) as described in Fig. 4, and the anti-CD3/CD28-induced $[Ca^{2+}]_i$ response of splenocytes was measured by fluorometry. A representative set of $[Ca^{2+}]_i$ responses is shown in the figure after subtracting the basal (unstimulated) values for $[Ca^{2+}]_i$

immunized with SRBC 4 days before the sacrifice, and spleen cells were tested for the anti-SRBC AFC response. Until 1 week after IL-1β treatment, there was no significant difference in the AFC response between controls and IL-1β-treated animals (data not shown); however, at 2 weeks after IL-1β treatment, ICV but not s.c. administration of IL-1β led to a significant decrease in the anti-SRBC AFC response (Fig. 6). ICV administration of IL-1β also inhibited T cell proliferation in response to Con A (Fig. 7A) and anti-TCR/CD28 (Fig. 7B). Moreover, the increase in $[Ca^{2+}]_i$ in response to TCR ligation was also attenuated by the IL-1β treatment (Fig. 7C). None of these parameters were affected by s.c. administration of IL-1β (not shown). These results suggest that chronic presence of low levels

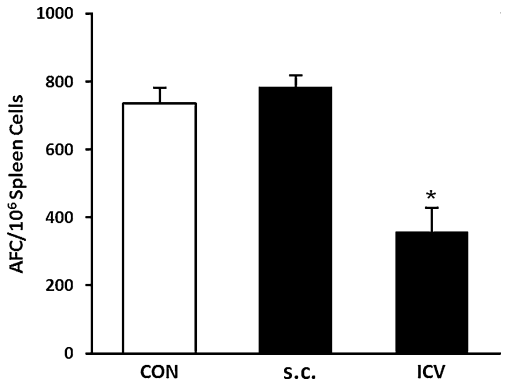


Fig. 6 Chronic ICV IL-1β, but not s.c. IL-1β treatment inhibits the anti-SRBC AFC response. Rats (n=6/group) were given IL-1β (100 ng/day/kg), aCSF (CON) through ICV-implanted cannulas, or IL-1β (s.c.; 100 ng/day/kg) via s.c. miniosmotic pumps for 2 weeks, and immunized with SRBC 4 days prior to sacrifice. The anti-SRBC AFC response of spleen cells was determined as described in Fig. 4. The graph represents mean ± SEM of AFC/10^6 spleen cells. * $P \leq 0.05$

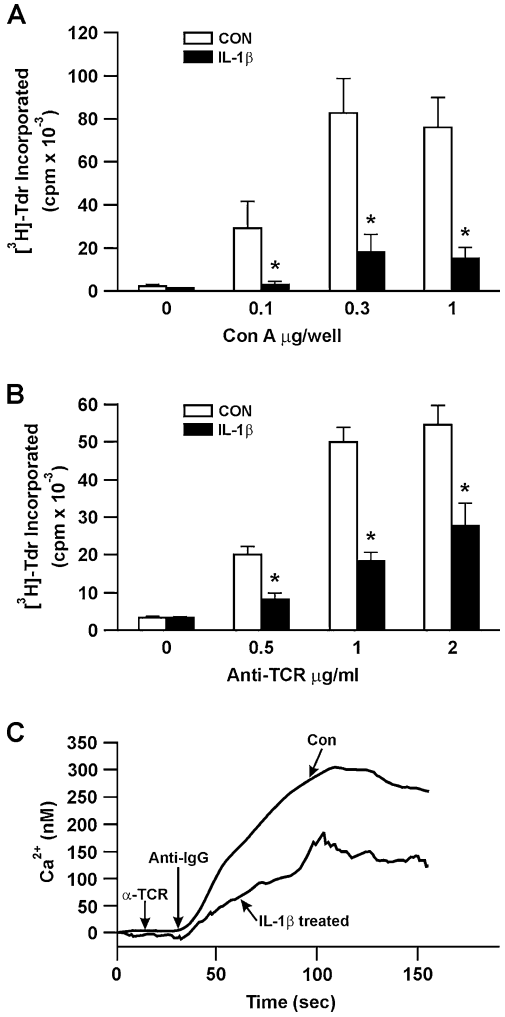


Fig. 7 Chronic ICV exposure to IL-1β causes immunosuppression. Rats (n=6/group) were treated with IL-1β or aCSF(CON) for 2 weeks as described in Fig. 6. Spleen cells were evaluated for Con A (**A**) and anti-TCR-induced (**B**) proliferative responses. (**C**) A representative profile of the spleen cell anti-TCR-induced $[Ca^{2+}]_i$ response is shown as described in Fig. 5. Bar graphs are mean ± SEM. * $P \leq 0.05$

of IL-1β in the brain, but not in the periphery, suppresses T cell responses.

Chronic IL-1β exposure does not increase serum CORT levels

Acute administration of IL-1β activates the hypothalamus-pituitary-adrenal (HPA) axis and increases the production of glucocorticoids (Skurlova et al. 2006). Therefore, it was possible that chronic low-dose IL-1β also stimulated CORT production that in turn suppressed T cell function. Blood CORT levels were determined in rats at 4 h after acute ICV IL-1β (50 ng/animal) administration and at 14 days after chronic ICV IL-1β (100 ng/day/kg body wt) exposure. While acute bolus exposure significantly increased the serum CORT level, chronic exposure

Author's personal copy

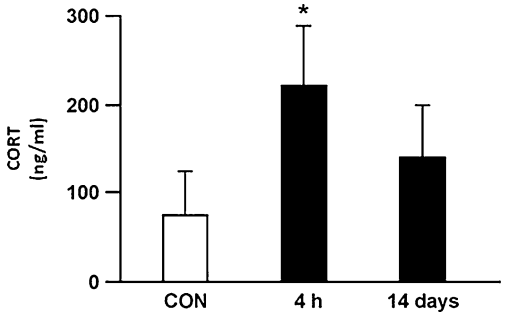


Fig. 8 Changes in serum CORT levels after ICV IL-1β administration. Rats were treated ICV with IL-1β (50 ng/day/kg) or aCSF (CON) for 4 h or 12 days. The 10:00 AM serum CORT levels were determined as described in Materials and methods. Bar graphs represent mean ± SEM of 4 animals/group. * $P \leq 0.05$

to IL-1β did not cause significant change in CORT level on day 14 (Fig. 8). Therefore, as with chronic NT treatment (Singh et al. 2000), it is unlikely that the immunosuppressive effects of the chronic IL-1β administration resulted from the increased serum CORT levels through activation of the HPA axis.

Chronic ICV administration of IL-1β reduces its pyrogenic activity

Chronic NT treatment blunts the turpentine-induced fever response in rats (Razani-Boroujerdi et al. 2004). To evaluate whether chronic ICV administration of IL-1β altered the pyrogenic response of animals, rats were intra-abdominally implanted with biotelemeters to monitor Tb. Rats were also implanted with ICV cannulas for chronic administration of aCSF (control) or IL-1β (100 ng/day/kg body wt) through s.c.-implanted miniosmotic pumps. Tb was recorded every 5 min, and average Tb traces for controls and IL-1β-treated rats during the light/dark cycle of the day (where the rats are at rest or active, respectively) are shown in Fig. 9. It is clear that during the early phase, IL-1β increased the Tb significantly and skewed the Tb circadian rhythm; however, continued presence of IL-1β blunted the Tb response and normalized its circadian rhythm. By day 10, IL-1β-treated rats were essentially indistinguishable from the aCSF-treated animals. To ascertain whether the IL-1β contained in the miniosmotic pumps retained its biological (pyrogenic) activity, pumps were removed from several animals on day 14, and the pooled material (equivalent to approximately 100 ng of IL-1β/kg body wt) was injected i.v. into naïve rats. Compared to aCSF-treated rats, the pooled sample caused a significant rise in Tb within 60 min of the injection (not shown), thus indicating that the IL-1β within osmotic pumps was biologically active at 2 weeks after the implantation. These results suggest that the constant presence of IL-1β in the brain blunts its pyrogenic activity.

Acute ICV administration of IL-1β activates Fyn and PTK activities in splenic T cells

One of the earliest effects of TCR ligation is the activation of PTK, including Src-like kinases Fyn and Lck (Salmond et al. 2009). NT exposure activates PTK and Fyn in T cells (Geng et al. 1996; Kalra et al. 2004). To determine whether IL-1β stimulates PTK activities, rats were surgically implanted with ICV cannulas, and 1 week after the surgery a single administration of 50 ng of IL-1β in 5 μl of aCSF or aCSF alone was injected into the cannulas. In another group of rats, 50 ng of IL-1β in 50 μl of PBS was injected i.v. Animals were sacrificed 2 h later, and splenic T cells were isolated. After culturing with anti-TCR or isotype control antibodies for 2 min, T cell lysates were prepared, run on gels, and probed with anti-phosphotyrosine antibodies to detect total PTK activity. Extracts were also treated with anti-Fyn monoclonal antibody, and the immunoprecipitated Fyn was assayed for the kinase activity. Prior to anti-TCR treatment, the basal PTK activity in T cells from control rats (aCSF-treated) was low; the activity increased significantly after the anti-TCR treatment (Fig. 10A). On the other hand, the PTK activity in T cells from ICV IL-1β-treated rats was high even before the anti-TCR treatment, and this activity did not increase significantly after the anti-TCR treatment. Similarly, the ICV IL-1β administration also increased the Fyn activity (Fig. 10B). These results suggest that within 2 h a single ICV but not i.v. exposure of IL-1β activates intracellular signaling in splenic T cells.

Discussion

We have previously shown that chronic ICV exposure to very small amounts of NT (human equivalent of <0.5 cigarettes/day) caused immunosuppression that was blocked by the non-selective nicotinic acetylcholine receptor (nAChR) antagonist mecamylamine (Singh et al. 2000). This suggested that central nAChRs are involved in the NT-induced immunosuppression. Moreover, NT treatment also blocked the pyrogenic response of turpentine (an inducer of brain IL-1β levels), suggesting a possible link between IL-1β (the major pyrogen) and immunosuppression. Similar effects of NT on immunosuppression and pyrogenic activity were also observed when NT was chronically administered s.c. at higher doses (human equivalent of about 1 pack/day) (Sopori et al. 1998a; Sopori et al. 1998b). Therefore, we expected that NT treatment would inhibit the formation of pyrogenic cytokine IL-1β in the brain. Surprisingly, however, the data presented herein clearly indicate that acute NT exposure actually increased IL-1β expression in the brain, and it is only after chronic NT exposure that IL-1β expression decreased to control levels. Although not as

Author's personal copy

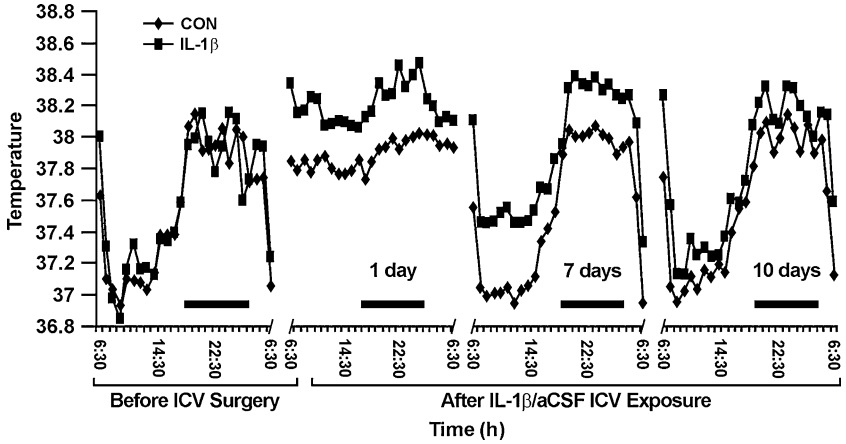


Fig. 9 Changes in Tb after ICV IL-1β or aCSF (CON) exposure. Rats (n=12/group) were implanted with biotelemeters to monitor Tb as described in Materials and methods. Seven days post-implantation, animals were given IL-1β (100 ng/d/kg) or aCSF (CON) via ICV cannulas in the morning. Average changes in Tb are shown for CON and IL-1β-treated animals 1 day before IL-1β/aCSF administration and 1, 7, and 10 days post IL-1β/aCSF ICV exposure. The horizontal bar over the X-axis indicates the dark (*active*) phase of the light/dark cycle

strongly, NT moderately increased the expression of TNF-α and IL-6 (not shown). Moreover, approximately 2 weeks into NT exposure, when challenged with an inflammatory stimulus such as turpentine or cryptococcal extracts (unpublished observation), the animals failed to elicit a proinflammatory cytokine response in the brain; however, the response to LPS was dose dependent (i.e., at low concentration of LPS nicotine was effective in blocking inflammation, but at high concentration, the selected concentration of nicotine was not very effective in moderating the proinflammatory response). Thus, NT induces a biphasic response in the brain: Acutely it induces the expression of proinflammatory cytokines, particularly IL-1β and, chronically, the anti-inflammatory phase replacing the initial proinflammatory phase, where continued presence of nicotine is unable to sustain the initial rise in IL-1β levels.

Proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 are produced during acute inflammatory responses (Dinarello 2000); however, IL-1β has also been implicated in neuroprotection (Stoll et al. 2002; Pinteaux et al. 2009). Increasing evidence shows that the early presence of these cytokines promotes protection against inflammation and sepsis (del Rey and Besedovsky 2000; Kox et al. 2000; Jin et al. 2009). In fact, when given prophylactically, IL-1β was protective in a mouse model of acute lung injury (Torre et al. 1994). Similarly, pretreatment with sublethal doses of endotoxin increases proinflammatory cytokine production, but it also protects against septic peritonitis through a mechanism independent of the adaptive immunity (Urbaschek and Urbaschek 1987; Varma et al. 2005); similar results are achieved by pretreatment with IL-1β and TNF-α (Urbaschek and Urbaschek 1987). Thus, in general, early presence of proinflammatory cytokines ushers in an anti-inflammatory phase, and it is possible that the early induction of IL-1β is critical for the subsequent immunosuppression and the anti-inflammatory response seen in chronically NT-treated animals.

Although, NT induces the expression of other proinflammatory cytokines (e.g., TNF-α, IL-6) in the brain, the predominant response is that of IL-1β, which is also implicated in the protection from neuroinflammation (Jin et al. 2009). To ascertain whether IL-1β is critical for the NT-induced immunosuppression, we used IL-1R KO mice. These mice tend to be immunologically hyporesponsive such as antibody response, clearance of pathogens (Lebeis et al. 2009), and pyrogenic effects of turpentine (Kozak et al. 1998); however, unlike the WT mice, chronic exposure of IL-1R KO mice to NT did not cause the progressive decline in the IL-1β expression in the brain. Moreover, IL-1R KO mice were refractory to the immunosuppressive effects of chronic NT treatment, such as antibody response to SRBC and anti-TCR/CD28-induced T cell proliferation. Although the KO animals also expressed TNF-α, it did not replace the function of IL-1β in inducing the anti-inflammatory phase of chronic NT treatment. Thus, the interaction between IL-1β and its receptor is critical for induction of NT-induced immunosuppression.

To prove that brain IL-1β modulates the immune/inflammatory responses, we examined the effects of ICV administration of IL-1β on various parameters of inflammation and TCR signaling. Initially, IL-1β increased fever and the serum concentration of CORT; however, continued presence of IL-1β prompted a progressive decrease in Tb and serum CORT levels, and at around 10 days after IL-1β treatment, Tb and CORT were comparable to control animals. The decrease did not reflect the loss of IL-1β biological activity in the implanted pumps; as the IL-1β collected on day 14 from the implanted pumps induced a fever response in naïve animals. In addition to down-

Author's personal copy

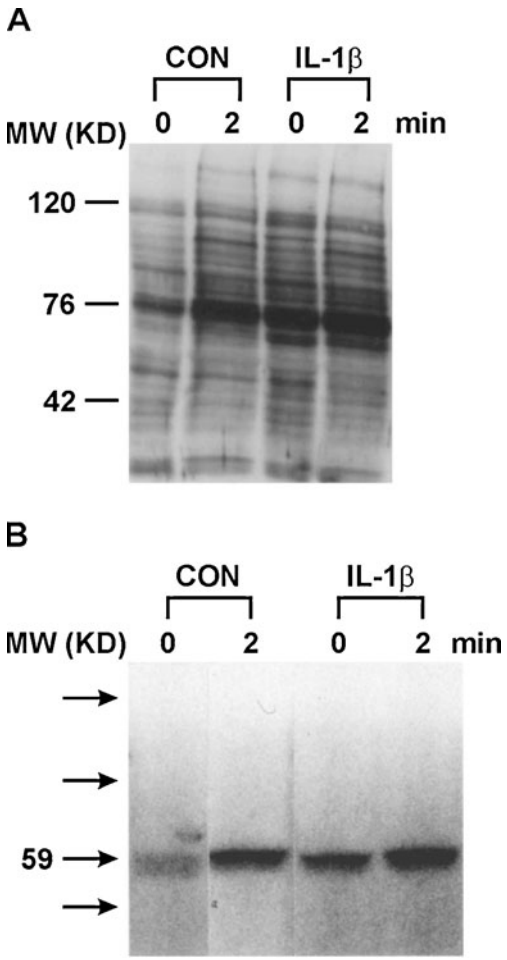


Fig. 10 Acute ICV administration of IL-1β activates Fyn and PTK activities in splenic T cells. Rats (3/group) were implanted with ICV cannulas 1 week prior to single injection of 5 μl of aCSF (CON) or IL-1β (50 ng/kg). Spleen cells were isolated and the extracts were resolved by Western blotting and visualized by anti-phosphotyrosine antibodies for PTK activity (**A**). The extracts were also immunoprecipitated with an anti-Fyn antibody, and the immunoprecipitates were used to determine Fyn kinase activity (**B**) as described in Materials and methods. Representative blots are shown in the figures

regulation of the acute phase response (i.e., activation of the HPA axis and fever response), chronic IL-1β exposure also suppressed the T cell function including the mitogen and TCR-induced proliferative responses, and production of antibodies to SRBC. Thus, chronic exposure to IL-1β attenuates the innate and adaptive immune responses.

Under our conditions, unlike ICV administration, chronic s.c. administration of low concentrations of IL-1β did not significantly affect the immune responses. This was surprising because both IL-1β and TNF-α are protective in the mouse sepsis model (Urbaschek and Urbaschek 1987). It is possible that, similar to NT (Sopori et al. 1998b), the concentration of IL-1β required to elicit a biological response through peripheral routes is much higher than the concentration needed to produce a similar response via the ICV route, and it is likely that in our experiments the concentration of IL-1β required to affect immune responses was not attained through s.c. administration.

Chronic NT failed to suppress T cell responses in IL-1R KO mice. Given that these KO mice lack a functional IL-1 receptor, the loss of IL-1 function in these animals is understandable; however, IL-1R KO mice have a functional TNF-α receptor and exhibit a moderate increase in TNF in response to NT (not shown). Thus, TNF-α is not as efficient as IL-1β in inducing the immunosuppressive phase in these mice. There are two potential but not necessarily mutually exclusive explanations for the failure of IL-1R KO mice to respond to elevated levels of TNF-α after NT treatment: (a) the immune/inflammatory responses in IL-1R KO are relatively weak and the concentration of TNF-α elicited by NT in KO mice was not sufficient to affect the immune response. Moreover, IL-6 and TNF-α are considered to be IL-1-responsive genes and are significantly reduced in IL-1R KO glial cells (Parker et al. 2002); (b) the efficacy of a cytokine to induce the protective response depends on the mouse strain. Thus, IL-1 is far superior in providing radioprotection in C57BL/6 than C3H mice, and TNF is better than IL-1 in C3H than C57BL/6 (Neta et al. 1988). Because IL-1R KO mice are on a C57BL/6 background, it is likely that IL-1β is a superior inducer than TNF-α in these mice. Although we directly tested only IL-1β in this model, NT also induced IL-1α and, because both cytokines activate the same receptor, it is possible that either of them could be effective in dampening the immune response.

A bidirectional communication exists between the brain and the immune system (Blalock 1994), and chronic presence of IL-1β in the brain may modulate this communication. However, it is clear that even the acute presence of IL-1β affected the interaction between the brain and the immune system and ICV, but not i.v., administration of relatively small concentrations of IL-1β-induced Fyn and PTK activities in splenic T cells within 2 h of the administration. Thus, changes in the brain milieu are immediately transmitted to the immune system.

The above results, we believe, provide the first direct demonstration that a neuroactive substance such as NT invokes a proinflammatory response in the brain to control the immune system. A number of human conditions are associated with upregulated expression of IL-1 in the brain that eventually leads to immunosuppression. For example, head trauma stimulates an inflammatory response followed by a compensatory anti-inflammatory response (Lenz et al. 2007; Lu et al. 2009). Similarly, the proinflammatory phase of neuroinflammation is essential for limiting subsequent inflammation (Kox et al. 2000; Varma et al. 2005), and we suggest that change in the cytokine milieu in the brain is an

Author's personal copy

important mechanism for limiting the immune/inflammatory responses. Furthermore, we postulate that the brain senses inflammation through proinflammatory cytokines, in particular IL-1, and, because inflammation can have severe adverse consequences in the CNS, the brain has developed a mechanism(s) to limit the immune and inflammatory responses. The precise mechanism by which the chronic presence of IL-1 limits immune/inflammatory responses is not clear. It is possible that the chronic presence of IL-1 downregulates the expression of IL-1 receptors and/or the production of the soluble IL-1R antagonist; however, these possibilities remain to be explored.

Acknowledgements The authors would like to thank Mr. Steve Randock and Ms. Paula Bradley for their help with graphics and editing, respectively.

Disclosures The authors have no financial conflict of interest.

References

Badjatia N (2009) Hyperthermia and fever control in brain injury. Crit Care Med 37:S250–257

Blalock JE (1994) The syntax of immune-neuroendocrine communication. Immunol Today 15:504–511

Cooper JA, Esch FS, Taylor SS, Hunter T (1984) Phosphorylation sites in enolase and lactate dehydrogenase utilized by tyrosine protein kinases in vivo and in vitro. J Biol Chem 259:7835–7841

del Rey A, Besedovsky HO (2000) The cytokine-HPA axis circuit contributes to prevent or moderate autoimmune processes. Z Rheumatol 59(Suppl 2):II/31–35

Dinarello CA (2000) Proinflammatory cytokines. Chest 118:503–508

Geng Y, Savage SM, Razani-Boroujerdi S, Sopori ML (1996) Effects of nicotine on the immune response. II. Chronic nicotine treatment induces T cell anergy. J Immunol 156:2384–2390

Geng Y, Savage SM, Johnson LJ, Seagrave J, Sopori ML (1995) Effects of nicotine on the immune response. I. Chronic exposure to nicotine impairs antigen receptor-mediated signal transduction in lymphocytes. Toxicol Appl Pharmacol 135:268–278

Gould KL, Hunter T (1988) Platelet-derived growth factor induces multisite phosphorylation of pp 60c-src and increases its protein-tyrosine kinase activity. Mol Cell Biol 8:3345–3356

Holt PG, Keast D (1977) Environmentally induced changes in immunological function: acute and chronic effects of inhalation of tobacco smoke and other atmospheric contaminants in man and experimental animals. Bacteriol Rev 41:205–216

Horai R, Asano M, Sudo K, Kanuka H, Suzuki M, Nishihara M, Takahashi M, Iwakura Y (1998) Production of mice deficient in genes for interleukin (IL)-1alpha, IL-1beta, IL-1alpha/beta, and IL-1 receptor antagonist shows that IL-1beta is crucial in turpentine-induced fever development and glucocorticoid secretion. J Exp Med 187:1463–1475

Jin W, Wang H, Yan W, Zhu L, Hu Z, Ding Y, Tang K (2009) Role of Nrf2 in protection against traumatic brain injury in mice. J Neurotrauma 26:131–139

Kalra R, Singh SP, Savage SM, Finch GL, Sopori ML (2000) Effects of cigarette smoke on immune response: chronic exposure to cigarette smoke impairs antigen-mediated signaling in T cells and depletes IP3-sensitive Ca(2+) stores. J Pharmacol Exp Ther 293:166–171

Kalra R, Singh SP, Pena-Philippides JC, Langley RJ, Razani-Boroujerdi S, Sopori ML (2004) Immunosuppressive and anti-inflammatory effects of nicotine administered by patch in an animal model. Clin Diagn Lab Immunol 11:563–568

Kox WJ, Volk T, Kox SN, Volk HD (2000) Immunomodulatory therapies in sepsis. Intensive Care Med 26(Suppl 1):S124–128

Kozak W, Kluger MJ, Soszynski D, Conn CA, Rudolph K, Leon LR, Zheng H (1998) IL-6 and IL-1 beta in fever. Studies using cytokine-deficient (knockout) mice. Ann N Y Acad Sci 856:33–47

Lebeis SL, Powell KR, Merlin D, Sherman MA, Kalman D (2009) Interleukin-1 receptor signaling protects mice from lethal intestinal damage caused by the attaching and effacing pathogen Citrobacter rodentium. Infect Immun 77:604–614

Lenz A, Franklin GA, Cheadle WG (2007) Systemic inflammation after trauma. Injury 38:1336–1345

Lu J, Goh SJ, Tng PY, Deng YY, Ling EA, Moochhala S (2009) Systemic inflammatory response following acute traumatic brain injury. Front Biosci 14:3795–3813

Mishra NC, Rir-Sima-Ah J, Langley RJ, Singh SP, Pena-Philippides JC, Koga T, Razani-Boroujerdi S, Hutt J, Campen M, Kim KC, Tesfaigzi Y, Sopori ML (2008) Nicotine primarily suppresses lung Th2 but not goblet cell and muscle cell responses to allergens. J Immunol 180:7655–7663

Neta R, Oppenheim JJ, Douches SD (1988) Interdependence of the radioprotective effects of human recombinant interleukin 1 alpha, tumor necrosis factor alpha, granulocyte colony-stimulating factor, and murine recombinant granulocyte-macrophage colony-stimulating factor. J Immunol 140:108–111

Parker LC, Luheshi GN, Rothwell NJ, Pinteaux E (2002) IL-1 beta signalling in glial cells in wildtype and IL-1RI deficient mice. Br J Pharmacol 136:312–320

Pinteaux E, Trotter P, Simi A (2009) Cell-specific and concentration-dependent actions of interleukin-1 in acute brain inflammation. Cytokine 45:1–7

Razani-Boroujerdi S, Sopori ML (2007) Early manifestations of NNK-induced lung cancer: role of lung immunity in tumor susceptibility. Am J Respir Cell Mol Biol 36:13–19

Razani-Boroujerdi S, Savage SM, Sopori ML (1994a) Alcohol-induced changes in the immune response: immunological effects of chronic ethanol intake are genetically regulated. Toxicol Appl Pharmacol 127:37–43

Razani-Boroujerdi S, Partridge LD, Sopori ML (1994b) Intracellular calcium signaling induced by thapsigargin in excitable and inexcitable cells. Cell Calcium 16:467–474

Razani-Boroujerdi S, Singh SP, Knall C, Hahn FF, Pena-Philippides JC, Kalra R, Langley RJ, Sopori ML (2004) Chronic nicotine inhibits inflammation and promotes influenza infection. Cell Immunol 230:1–9

Razani-Boroujerdi S, Boyd RT, Davila-Garcia MI, Nandi JS, Mishra NC, Singh SP, Pena-Philippides JC, Langley R, Sopori ML (2007) T cells express alpha7-nicotinic acetylcholine receptor subunits that require a functional TCR and leukocyte-specific protein tyrosine kinase for nicotine-induced Ca2+ response. J Immunol 179:2889–2898

Salmond RJ, Filby A, Qureshi I, Caserta S, Zamoyska R (2009) T-cell receptor proximal signaling via the Src-family kinases, Lck and Fyn, influences T-cell activation, differentiation, and tolerance. Immunol Rev 228:9–22

Scott DA, Martin M (2006) Exploitation of the nicotinic anti-inflammatory pathway for the treatment of epithelial inflammatory diseases. World J Gastroenterol 12:7451–7459

Singh SP, Kalra R, Puttfarcken P, Kozak A, Tesfaigzi J, Sopori ML (2000) Acute and chronic nicotine exposures modulate the immune system through different pathways. Toxicol Appl Pharmacol 164:65–72

Author's personal copy

Skurlova M, Stofkova A, Jurcovicova J (2006) Exogenous IL-1beta induces its own expression, but not that of IL-6 in the hypothalamus and activates HPA axis and prolactin release. Endocr Regul 40:125–128

Sopori M (2002) Effects of cigarette smoke on the immune system. Nat Rev Immunol 2:372–377

Sopori ML, Cherian S, Chilukuri R, Shopp GM (1989) Cigarette smoke causes inhibition of the immune response to intratracheally administered antigens. Toxicol Appl Pharmacol 97:489–499

Sopori ML, Kozak W, Savage SM, Geng Y, Kluger MJ (1998a) Nicotine-induced modulation of T Cell function. Implications for inflammation and infection. Adv Exp Med Biol 437:279–289

Sopori ML, Kozak W, Savage SM, Geng Y, Soszynski D, Kluger MJ, Perryman EK, Snow GE (1998b) Effect of nicotine on the immune system: possible regulation of immune responses by central and peripheral mechanisms. Psychoneuroendocrinology 23:189–204

Stampfli MR, Anderson GP (2009) How cigarette smoke skews immune responses to promote infection, lung disease and cancer. Nat Rev Immunol 9:377–384

Stoll G, Jander S, Schroeter M (2002) Detrimental and beneficial effects of injury-induced inflammation and cytokine expression in the nervous system. Adv Exp Med Biol 513:87–113

Torre D, Minoja G, Maraggia D, Chiaranda M, Tambini R, Speranza F, Giola M (1994) Effect of recombinant IL-1 beta and recombinant gamma interferon on septic acute lung injury in mice. Chest 105:1241–1245

Ulloa L, Wang P (2007) The neuronal strategy for inflammation. Novartis Found Symp 280:223–233, discussion 233–227

Urbaschek R, Urbaschek B (1987) Tumor necrosis factor and interleukin 1 as mediators of endotoxin-induced beneficial effects. Rev Infect Dis 9(Suppl 5):S607–615

van Westerloo DJ, Giebelen IA, Florquin S, Daalhuisen J, Bruno MJ, de Vos AF, Tracey KJ, van der Poll T (2005) The cholinergic anti-inflammatory pathway regulates the host response during septic peritonitis. J Infect Dis 191:2138–2148

Varma TK, Durham M, Murphey ED, Cui W, Huang Z, Lin CY, Toliver-Kinsky T, Sherwood ER (2005) Endotoxin priming improves clearance of Pseudomonas aeruginosa in wild-type and interleukin-10 knockout mice. Infect Immun 73:7340–7347

Wang RD, Tai H, Xie C, Wang X, Wright JL, Churg A (2003) Cigarette smoke produces airway wall remodeling in rat tracheal explants. Am J Respir Crit Care Med 168:1232–1236

Title: Gestational Exposure to Secondhand Cigarette Smoke Causes Bronchopulmonary Dysplasia Blocked by Mecamylamine

Running title: Gestational smoke induces bronchopulmonary dysplasia

Authors: Shashi P. Singh[a], Sravanthi Gundavarapu[a], Kevin R. Smith[a], Ali Imran Saeed[b], Neerad C. Mishra[a], Edward G. Barrett[a], Matloob Husain[c], Hitendra S. Chand[a], Kevin S. Harrod[a], Raymond J. Langley[a], and Mohan L Sopori[a]

Affiliations: [a]Lovelace Respiratory Research Institute, Albuquerque, NM 87108, USA; [b]Pulmonary and Critical Care Medicine, University of New Mexico, Albuquerque, NM 87131, USA; [c]Department of. Microbiology and Immunology, University of Otago, Dunedin, New Zealand 9016

Corresponding Author:

Mohan Sopori, Ph.D.

Immunology Division, Lovelace Respiratory Research Institute

Albuquerque, NM 87108

Tel: (505) 348-9440

Fax: (505) 348-4986

Email: msopori@lrri.org

This research was supported in part by a grant from US Army Medical Research and Material Command (GW093005), Flight Attendant Medical Research Institute grants to S.P.S. and N.C.M., and funds from Lovelace Respiratory Research Institute (IMMSPT).

Abstract:
Background: Gestational exposure to cigarette smoke (CS) through parental smoking adversely affects pulmonary function in children, and increases the risk of bronchopulmonary dysplasia (BPD). BPD is a neonatal lung condition characterized by decreased alveolarization, angiogenesis and surfactant production; however, the mechanism by which gestational exposure to CS induces BPD is not clear.

Objective: To determine if gestational CS induces BPD in mice and ascertain the role of nicotinic acetylcholine receptors in this response.

Methods: BALB/c or C57BL/6 mice were exposed to fresh air (control) or secondhand CS (SS) throughout gestation or postnatally up to 10 wk. Lungs were examined 7 days, 10 weeks, and 8 months afterbirth.

Results: Gestational, but not postnatal exposure to SS caused typical BPD-like condition associated with suppressed angiogenesis (decreased VEGF, VEGFR, and CD34), irreversible hypoalveolarization, and significantly decreased expression of Clara cell secretory protein and surfactant protein-B without affecting airway ciliated cells. Importantly, treatment with nicotinic receptor antagonist (mecamylamine) during the gestational period blocked the effects of SS-induced structural changes in the lung and improved the lung secretory function.

Conclusions: Gestational exposure to SS irreversibly disrupts lung development leading to BPD-like condition with hypoalveolarization, decreased angiogenesis and diminished lung secretory function. Nicotinic receptors are critical in the induction of gestational SS-induced BPD, and use of nicotinic receptor antagonists during pregnancy may block the development of gestational CS-induced BDP. (**Word Count: 227**)

Clinical Implications:

Many prospective mothers smoke during pregnancy, a risk for BDP in children. Administration of mecamylamine during pregnancy blocks BPD in mice. Thus nicotinic receptor antagonists have therapeutic potential in humans.

Capsule summary:

Gestational exposure to cigarette smoke causes BPD associated with decreased angiogenesis, decreased surfactant/secretory proteins, and irreversible hypoalveolarization. Presence of the nicotinic acetylcholine receptor antagonist mecamylamine during pregnancy blocks cigarette smoke-induced BPD and restores lung function.

Key words:

Cigarette Smoke; Bronchopulmonary Dysplasia; Secretory/Surfactant proteins; Angiogenesis; Nicotinic Receptor Antagonists

Symbols, Abbreviations, and Acronyms

BPD	Bronchopulmonary dysplasia
CCSP	Clara cell secretory protein
COPD	Chronic obstructive pulmonary disease
CS	Cigarette smoke
FA	Filtered air (control)
H&E	Hematoxylin and eosin
IHC	Immunohistochemical
L_m	Mean linear intercept
MMP	Metalloproteinase
nAChR	Nicotinic acetylcholine receptor
PBS	Phosphate-buffered saline
qPCR	Quantitative polymerase chain reaction
RT	Room temperature
SP-B	Surfactant protein-B
SS	Secondhand cigarette smoke

INTRODUCTION

Bronchopulmonary dysplasia (BPD), first described in 1967 in borderline premature infants with respiratory distress syndrome (1) was characterized by fewer and enlarged alveoli, suppressed angiogenesis, lack or insufficient production of surfactant proteins (SP), and lung inflammation (2). However, impaired alveolar development is the main pathophysiological manifestation of BPD (3). Twenty to 40% of prematurely and low-birth-weight (<28 weeks of gestation and under 1 kg body weight) infants develop BPD (4, 5), and it is the second leading cause of mortality among the infants born before 28 weeks of gestation (6). Multiple factors that injure the immature lung contribute to the development of BPD, including exposure of newborn infants to supplemental oxygen, barotrauma, neonatal infections, ductus arteriosus, and inflammation (7).

Exposure to a wide range of chemicals and environmental toxicants during perinatal life significantly affects the maturation and function of the respiratory system (8). Lung injuries during canalicular and saccular phases affect alveolar and vascular development of the lung and promote BPD (9), and antenatal factors that disrupt lung development may impair respiratory function in adulthood (10). While adult exposure to tobacco smoke is a major risk factor for respiratory diseases including chronic obstructive pulmonary disease (COPD) (11), the risk of cigarette smoke (CS)-associated pulmonary complications is highest during fetal and early postnatal life (12, 13), yet nearly one third of prospective mothers smoke during some stages of pregnancy (14, 15). Epidemiological evidence and animal experiments suggest that parental, particularly maternal, smoking or nicotine exposure adversely affects the pulmonary health of the offspring, including higher risk for the development of allergic asthma, and airway remodeling and function (16, 17 18,19, 20). Gestational exposure to CS is also an independent risk factor for BPD and infants with BPD have a significantly higher risk of developing COPD and asthma later in life (21). The mechanism by which CS smoke promotes BPD is not clear. Experiments performed decades earlier indicated that CS causes fetal lung hypoplasia in mice

(22) and, more recent experiments suggest that intrauterine exposure to nicotine or CS decreases lung volumes in monkeys (16) and increases the dependence on oxygen at day 28 of life in human infants (21). In this communication, we show that gestational, but not early postnatal exposure of mice to secondhand cigarette smoke (SS) suppresses angiogenesis, alveolarization, and the development of Clara and goblet cells without causing significant lung inflammation, and the SS-induced effects on alveolar architecture are irreversible. However, concomitant exposure to the nicotinic acetylcholine receptor (nAChR) antagonist mecamylamine (MM) blocks the effects of gestational SS on lung function.

METHODS: A brief description of the methods is provided below; however, detailed methods are provided in the Supplemental section.

Animals, cigarette smoke generation and exposure: Pathogen-free adult (3-4 months old) BALB/c or C57BL/6 male and female mice (FCR Facility, Frederick, MD) were exposed to whole-body SS or fresh air (FA) for 6 hours/day, 7 days/week as described (23). This dose of SS approximates the smoke exposure a pregnant woman would receive by sitting in a smoking bar for 3 hr/day throughout the gestational period (17, 18, 23). Pregnant mice were separated, and continued to receive SS or FA until the pups were born. Immediately after the birth of pups smoke exposure was stopped; however, some gestationally FA exposed pups were exposed to SS until 10 wk afterbirth (postnatal SS). Prior to SS or FA exposure, some breeder mice were subcutaneously implanted with ALZET miniosmotic pumps containing saline or MM (2.5 mg/ml), generating FA (control), MM+FA, and MM+SS groups. Animals were sacrificed at indicated times.

Tissue preparation, immunostaining, and other assays: After sacrifice, lungs were removed, inflated and fixed at a constant hydrostatic pressure (24, 25). Where indicated, right lung was used for bronchoalveolar lavage (BAL) and then frozen for Western blot and qPCR analysis. Lung sections were stained with Hematoxylin & eosin (H&E) (18), and the mean linear intercept (L_m), a measure of interalveolar wall distance (26, 27), was determined. For immunohistochemistry (IHC) lung sections were treated with specific antibodies after antigen retrieval and blocking of endogenous peroxidases and non-specific reactivity. IHC and immunofluorescence techniques (28-30) were used to visualize and quantitate SP-B^+ and $CCSP^+$ (Clara cells), β-$tubulin^+$ (ciliated cells), $SPDEF^+$ (goblet cells) and $CD34^+$ (endothelial cells). To determine lung fibrosis, lung sections were stained with Masson's Trichrome (Sigma-Aldrich) to reveal collagen as described previously (31). Western blot analysis of lung homogenates and qPCR analysis of RNA were carried out as described previously (17, 18). Data were analyzed using Graph Pad Prism software 5.03. One-way ANOVA was used to

compare the mean between the groups using the Tukey post-hoc test. The student's t test was used for comparison between two groups. Results are expressed as the means ± SD.

RESULTS

Prenatal SS exposure suppresses alveolarization

Approximately 80% of alveolarization occurs postnatally and the development of alveoli in humans and mice continues until about 7 years and 4 weeks after birth, respectively (32). Hypoalveolarization is the hallmark of BPD (33), and increasing epidemiological evidence suggests that BPD-related changes in respiratory function may continue later in life (34, 35). To determine the effect of prenatal SS exposure on BPD, lung sections from mice exposed gestationally to FA (control) or SS were examined microscopically at 7 days, 10 weeks, and 8 months after birth. H&E staining of lung sections suggests that gestational exposure to SS caused areas of hypoalveolarization in the lung and was seen in the early lung (7 days) as well as at 10 weeks and 8 months after the birth (Fig. 1A, 1B, and 1C). Changes in lung septation were quantitated by determining the L_m of alveoli. L_m values show similar increases (approximately 25%) in gestationally SS-exposed lungs at 7 days, 10 weeks, and 8 months after the birth (Bar Graph Fig. 1A, 1B, and 1C, respectively). Moreover, histopathology of the alveolar septae suggests that instead of elongated secondary septa seen in control lungs, SS-exposed lungs have an increased presence of crested secondary septae, indicating incomplete secondary alveolar septal formation (Fig. 1, arrows)—a feature also seen in human BPD (36, 37). On the other hand, postnatal exposure to SS (day 1 through 10 weeks after the birth) did not cause a significant change in alveolarization; L_m values were comparable to control animals (Suppl. Fig. E1A). Thus, in utero but not early postnatal exposure to SS impairs lung septation and these SS-induced deficits in alveolarization are irreversible at least until 8 months of life in BALB/c mice. To ascertain whether BALB/c mice were unique in developing gestational SS-induced hypoalveolarization, we also exposed C57BL/6 mice to gestational SS. Results from SS-exposed 7-day lung from C57BL/6 lung (Suppl. Fig. E1B) show morphometric changes similar to those in BALB/c mice. Thus both BALB/c and C57BL/6 mice develop BPD in response to gestational SS.

In addition to impaired postnatal lung growth (38, 39), BPD may cause variable interstitial fibrosis (39); however, trichrome staining did not show any significant difference in collagen content between FA and SS-exposed 10-wk old lungs (Suppl. Fig. E2). Moreover, there was no indication of leukocytic infiltration in gestationally SS-exposed lungs (not shown). These results suggest that the SS-induced BPD is not associated with significant lung fibrosis or inflammation.

Gestational SS exposure impairs the development of Clara and goblet cells but not of ciliated epithelium cells

Downregulation of secretory proteins contributes to the pathology of several airway diseases, including BPD (40, 41), and many premature infants are given a mixture of SP-B and SP-C (surfactant proteins) to improve survival (39). We have observed that mice exposed gestationally to SS have significantly lower allergen-induced Muc5ac expression and airway mucus formation (18). To determine whether gestational SS broadly affected the airway secretory functions, we examined airway Clara and goblet cells in FA and SS-exposed mouse lung sections at 7 days post birth by staining for CCSP and SP-B (Clara cell markers) and SPDEF (goblet cell marker). As seen in Fig. 2A (CCSP), Fig. 2B (SP-B), and Fig. 2C (SPDEF), the presence of Clara and goblet cells is significantly reduced in mice gestationally exposed to SS. On the other hand, as scored by β-tubulin staining (Fig. 2D), no significant changes in the airway ciliated epithelial cells are seen between FA- and SS-exposed mice at 7 days afterbirth. The SS-induced changes in the secretory function (i.e., expression of CCSP and SP-B) were also determined by qPCR analysis and showed that SS exposure strongly decreased the mRNA expression of CCSP (Fig. $2E_a$) and SP-B (Fig. $2E_b$). On the other hand, quantitation of β-tubulin-positive cells/μm basal lamina did not show any significant changes between gestationally FA- and SS-exposed lungs (Fig. $2E_c$).Thus, as in human BPD, prenatal exposure to SS impairs the development of Clara and goblet cells; the reduction in these secretory cells may contribute to the reduced expression of surfactant proteins and airway mucus formation in gestationally SS-

exposed lungs. However, the SS exposure does not affect the development of airway ciliated epithelial cells.

Gestational SS impairs airway angiogenesis

Angiogenesis is a tightly regulated physiological process during embryogenesis (42) and plays a vital role in the development of lung and airways (43). To ascertain whether changes in alveolarization from gestational exposure to SS reflected impaired angiogenesis, lung vascularization was evaluated by IHC and immunofluorescence staining for CD34, a transmembrane glycoprotein on endothelial cells (44). Compared to controls, the SS-exposed lungs at 7 days after birth exhibited much weaker immunostaining (Fig. $3A_a$) and CD34 immunofluorescence (Fig. $3A_b$), indicating a decreased number of endothelial cells in gestationally SS-exposed lungs. Moreover, Western blots of the lung extracts from FA- and SS-exposed animals showed that SS-exposed lungs had significantly reduced levels of CD34 immunoreactive protein (Fig. 3B). Thus, gestational SS exposure strongly suppresses/impairs vascular development in the lung.

Coordinated and timely release of angiogenic growth factors from respiratory epithelial cells promotes normal alveolar development (45). Although multiple factors affect angiogenesis, VEGF plays a key role in morphogenic events during angiogenesis (46-50). The expression of VEGF receptors increases during lung development, and most of the VEGF effects are mediated through VEGFR2 (49, 51). We determined the expression of VEGFR2 by qPCR in the 7-day lung from FA- and SS-exposed animals. Consistent with CD34 results, VEGFR2 expression was significantly reduced in the lung samples from SS-exposed animals (Fig. 3C). Moreover, as determined by ELISA, the concentration of VEGF in BALF from SS-exposed mouse lungs at 10 weeks after birth was significantly lower than control lungs (Fig. 3D). These results suggest that gestational exposure to SS causes angiogenic defects in the developing

lung. Thus, decreased production of VEGF and the expression of its main receptor VEGFR2 might contribute to the angiogenic defects in gestationally SS-exposed animals, and these defects continue at least up to 10 weeks after birth.

Mecamylamine blocks SS-induced effects on lung pathology

There is evidence that prenatal exposure to nicotine affects lung development and function (14, 16, 52, 53). Therefore, it was possible that the effects of SS on lung development and function were regulated by nAChRs, and blocking these receptors would prevent the gestational SS-induced injury to the lung. To test this possibility, mice were implanted subcutaneously with saline- (control) or MM (nAChR antagonist)-containing miniosmotic pumps, delivering MM to pregnant mothers throughout the gestational period. We observed that while MM alone did not significantly affect alveolarization and L_m of 7-day old lung, it blocked the effects of gestational SS on alveolar septation (Fig. 4A) and L_m values (Fig. 4B). IHC and immunofluorescence staining (Fig. $4C_a$ and Fig. $4C_b$) of the lung section, and qPCR analysis (Fig. 4D) indicated that pretreatment with MM also normalized lung vascularization (CD34 expression) and the mRNA expression of VEGFR2 in the 7-day old lung. qPCR analysis also indicated that MM treatment blocked the gestational SS-induced reduction in SP-B (Fig. 5A) and CCSP (Fig. 5B). Similarly, MM prevented the SS-induced reduction in the airway SP-B staining (Fig. 5C) and CCSP staining (Fig. 5D). Moreover, quantitation of $SP\text{-}B^+$ and $CCSP^+$ cells/μm basal lamina showed that the MM treatment countered the effects of SS on $SP\text{-}B^+$ cells (Fig. $5E_a$) and $CCSP^+$ cells (Fig. $5E_b$) in the airways. Thus, MM treatment blocks the inhibitory effects of gestational SS on alveolarization, vascularization, and secretory/surfactant protein production, indicating a critical role of nAChRs in the development of SS-induced BPD.

DISCUSSION

Extensive epidemiologic and experimental evidence from studies in mice and humans demonstrate the detrimental long-term pulmonary outcomes in the offspring of mothers who smoke during pregnancy (19, 20). Increasing evidence suggests that *in utero* exposure to environmental toxins, including CS/nicotine, affects lung development and function (16, 18, 19, 20, 54). Although the development of fetal lung hypoplasia after maternal exposure of rats to CS was reported several decades ago (53), intrauterine smoke exposure as an independent risk factor for BPD in human infants was recognized relatively recently (21). BPD is a disease whose etiology has not been fully established and may arise from various types of insults to the immature lung (7). Herein we present evidence that gestational, but not early postnatal exposure of BALB/c and C57/BL6 mice to realistic doses of SS causes typical characteristics of BPD and, at 7 days afterbirth, and gestationally SS-exposed lungs exhibit reduced septation as determined by histopathologic analysis and significant increases in L_m values. L_m represents the changes in the acinar airspace complex and provides an estimate of the size and changes in air spaces (56); the average increase in L_m (≥23%) in SS-exposed lungs is comparable to some patients with COPD/emphysema (57).

Chronic exposure to CS is the most important cause of emphysema/COPD in humans (58), and the loss of alveolar surface is associated with increased concentrations of metalloproteinases (MMP) (58, 59). MMP-12 activity that degrades elastin and other components of extracellular matrix components has been implicated in humans and animal models of emphysema (60); however, MMP-12 was not elevated in SS-exposed animals (Singh et al., unpublished). It is likely that the major difference between classical emphysema and alveolar simplification in the SS-induced BPD is that the latter results from impaired formation of secondary alveolar septae (36) and, in the absence of additional lung insults, is not significantly progressive; whereas, the

classical emphysema is progressive and results from dissolution of preformed alveolar septae. Thus, gestational SS-induced changes in the alveolar structure may result primarily from the arrest in alveolar development rather than destruction of preformed alveolar septae in typical COPD/emphysema. The histopathology of SS-exposed 7-day old lung showing increased presence of crested secondary septa, indicated incomplete formation of secondary alveolar septa (36) that further supports this inference. These results suggest that, although multiple causes of lung injury, including lung inflammation might encourage development of BPD (61-64), the gestational SS-induced BPD results primarily from inhibition of normal lung maturation (65).

Interaction between epithelial and vascular compartments is critical in alveologenesis and coordinated and timely release of angiogenic growth factors from respiratory epithelial cells promotes normal alveolar development (45). Fewer alveoli and microvessels are quintessential features of neonatal chronic lung disease (66), and pulmonary endothelium is closely apposed to the developing epithelium (49). VEGF is the most potent mediator of angiogenesis (47-49) and plays a significant role in the pathophysiology of common respiratory disorders including acute lung injury, asthma, COPD, pulmonary fibrosis, and lung cancer (67), and the angiogenic effects of VEGF are primarily mediated through the VEGF receptors VEGFR2 (49, 51, 68). Consistent with the critical role of VEGF and VEGFR2 in BPD, the infants who die of BPD have little or no VEGF in their lung epithelium (25). BDP is also associated with decreased levels of angiogenic progenitor cells in cord blood (69) and reduction of VEGF receptors in pulmonary vasculature (45). VEGF and VEGFR2 are also significantly decreased in COPD/emphysema patients and in animals repeatedly exposed to CS (70). CD34 is present on endothelial cells, and is involved in leukocyte adhesion and endothelial cell migration during angiogenesis (71). To ascertain angiogenesis, we scored for CD34 expression by IHC, immunofluorescence and Western blot analysis. Compared to control, the expression of CD34 in the 7-day SS-exposed

lung was severely reduced. Lung expression of VEGF and/or VEGFR2 was also significantly decreased in the 10-week SS-exposed lungs and, compared to control the concentration of VEGF protein was lower in the BALF in these animals. In preterm children with BPD, VEGF increases over time but remains significantly lower than preterm children without BPD (72). Decreased expression of VEGFR2 was also seen by microarray analysis of carotid arteries in monkeys exposed during gestation and early postnatal life to environmental tobacco smoke (73). These results suggest that decreased alveolarization in gestationally SS-exposed lung may reflect the reduction in VEGF and VEGFR2 that impacts lung vascularization.

Another characteristic of BPD is decreased production of CCSP and other surfactant proteins (74). Additionally, CCSP modulates immune responses, reduces lung injury in animal models, upregulates the expression of surfactant proteins and VEGF in the lung, and ameliorates BPD (75). SP-B is an important surfactant protein that lowers surface tension and prevents atelectasis and protects epithelial cells (76, 77), and its administration improves clinical outcomes in BPD (78). The expression of both CCSP and SP-B is significantly downregulated in the gestationally SS-exposed 7-day lung. SS exposure also inhibits other airway epithelial cell functions, such as mucus formation (18). It is possible that gestational SS affected the overall development of airway epithelial cells and their function. There are three major epithelial cell types in the airways: ciliated cells, Clara cells, and goblet cells. The ciliated epithelium participates in the mucociliary clearance and expresses β-tubulin on the surface (29, 30), while Clara and goblet cells express CCSP and SPDEF, respectively. IHC analysis clearly indicated that gestational SS did not significantly affect ciliated (β-tubulin$^+$) cells, but a drastic reduction was noted in CCSP$^+$ and SPDEF$^+$ cells in the airways. In fact, it was difficult to visualize CCSP$^+$ and SPDEF$^+$ cells in the airways of gestationally SS-exposed animals. Thus, the severe lack of secretory proteins in SS-exposed lungs may reflect impaired/delayed development of these cells. Indeed, the impaired airway secretory function such as mucus formation (18) and CCSP/SP-B was evident even at 10 weeks after the birth of gestationally SS-exposed animals

(Suppl.E3A/B). Additionally, CCSP stimulates VEGF production (75, 79) and participates in alveologenesis (79). Thus, loss/decreased expression of CCSP may contribute to impaired angiogenesis and alveologenesis in gestationally SS-exposed lungs.

CS, including SS contains many toxic chemicals (80) and, in some respects, SS may be more toxic than mainstream CS (81). Nicotine can concentrate in the fetus (82), and the presence of nAChRs has been observed in number of non-neuronal cell types, including endothelial (83) and lung epithelial cells (84). Thus, it is possible that the effects of SS on lung alveolarization, angiogenesis, and surfactant proteins were mediated through nAChRs. Indeed, treatment with MM during the gestational period blocked the effects of gestational SS on septation, angiogenesis, and airway secretory function. These results clearly suggest that effects of *in utero* SS on lung development are mediated through nAChRs. A surprising observation was that the *in utero* SS effects on some lung parameters were opposite of those observed in adult mice after CS or nicotine exposure. For example, exposure of adult mice to CS/nicotine suppresses allergic responses (85), but prenatal exposure to SS strongly exacerbates allergen-induced atopy and Th2 polarization (18). Similarly, unlike its anti-angiogenic effects during gestational period, nicotine stimulates VEGF and promotes angiogenesis and neovascularization in the adult mice (83). Moreover, unlike the results from gestational SS exposures, nicotine treatment of adult mice promotes mucus formation in the airways (18). A potential explanation is that long-term exposure to low levels of nicotine may promote desensitization or loss of nAChRs in the lung, and these receptors may be important in regulating lung growth. Although currently we have no direct evidence to support this possibility, recent papers suggest that while short-term exposure to nicotine may promote angiogenesis and VEGF production, long-term exposures to nicotine may impair cholinergic angiogenesis (70, 86).

Although maternal smoking is an independent risk factor for BDP in children (21), BALB/c and C57BL/6 mice might be particularly sensitive to pro-BPD effects of gestational cigarette smoke.

Alternatively, unlike humans, experimental mice are inbred and the presence of susceptibility factors on both alleles might make them more susceptible to CS-induced BPD. Nonetheless, mice are likely to serve as an excellent animal model to study the mechanism by which nAChRs regulate lung development and BPD. Interestingly, postnatal SS had very little effect on the development of BPD. In view of recent human data suggesting that exposure to CS during first trimester is sufficient to increase the risk of asthma in children (20), CS-induced changes during early embryogenesis may also affect lung development and expression of BPD. Overall, the current study shows that exposure to tobacco smoke during gestation interferes with alveolarization and promotes a BPD-like syndrome. These effects of SS are mediated through nAChRs and antagonists of nAChRs may have therapeutic value in blocking the effects of CS/nicotine on fetal lung development.

Acknowledgements

The authors thank Dr. Julie Hutt (veterinary pathologist) for her help in reading microscopic slides, Thomas Gagliano for the graphics, Paula Bradley for editorial work, and Juan Carlos Pena-Philippides for implantation of miniosmotic pump.

List of Supplementary Materials

Detailed description of Methods

Suppl. Fig. E1. (A) Postnatal exposure to SS does not affect alveolarization; (B) C57BL/6 mice are also sensitive to gestational exposure to SS.

Suppl. Fig. E2. Lung collagen content is unaffected by gestational SS.

Suppl. Fig. E3. CCSP and SP-B are reduced in SS-exposed lungs at 10 wk afterbirth.

References

1. Northway Jr. WH, Rosan RC, Porter DY. Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. N. Engl. J. Med. 1967;276: 357-368.

2. Monte LF., Silva Filho LV, Miyoshi MH, Rozov T. Bronchopulmonary dysplasia. J. Pediatr. (Rio. J.). 2005;8:99-110.

3. American Thoracic Society Documents. Am. J. Resp. Crit. Care Med., 2003;168: 356-396.

4. Smith VC., Zupancic JA, McCormick MC, Croen LA, Greene J, Escobar GJ, et al., Rehospitalization in the first year of life among infants with bronchopulmonary dysplasia. J. Pediatr. 2004;144: 799-803.

5. Lima MR., Andrade MA, Araújo AP, Figueroa JN, Andrade LB. Influence of maternal and neonatal factors on bronchopulmonary dysplasia development. Rev. Assoc. Med. Bras. 2011;57: 391-396.

6. Callaghan WM., MacDorman MF, Rasmussen SA, Qin C, Lackritz EM. The contribution of preterm birth to infant mortality rates in the United States. Pediatrics 2006;118: 1566-1573.

7. Bancalari E, Claure N, Sosenko IR. Bronchopulmonary dysplasia: changes in pathogenesis, epidemiology and definition. Semin. Neonatol. 2003;8: 63-71.

8. Pinkerton KE, Joad JP. The mammalian respiratory system and critical windows of exposure for children's health. Environ. Health Perspect. Suppl. 2000;108 Suppl. 3: 457-460.

9. Merritt TA, Deming DD, Boynton BR. The 'new' bronchopulmonary dysplasia: challenges and commentary. Semin. Fetal Neonatal Med. 2009;14: 345-357.

10. Greenough, A. Prenatal factors in the development of chronic lung disease. Semin. Fetal Neonatal Med. 2009;14: 339-344.

11. Sopori M. Effects of cigarette smoke on the immune system. Nat. Rev. Immunol**.** 2002;2**:** 372-377.

12. Scott JE. The pulmonary surfactant: Impact of tobacco smoke and related compounds on surfactant and lung development. Tob. Induc. Dis. 2004;2: 3-25.

13. DiFranza JR., Aligne CA, Weitzman M. Prenatal and postnatal environmental tobacco smoke exposure and children's health. Pediatrics 2004;113: 1007-1015.

14. Sandberg K, Poole SD, Hamdan A, Arbogast P, Sundell HW. Altered lung development after prenatal nicotine exposure in young lambs. Pediatr. Res. 2004;56: 432-439.

15. Hylkema MN., Blacquière MJ. Intrauterine effects of maternal smoking on sensitization, asthma, and chronic obstructive pulmonary disease. Proc. Am. Thorac. Soc. 2009**;**6: 660-662.

16. Sekhon HS., Keller JA, Benowitz NL, Spindel ER. Prenatal nicotine exposure alters pulmonary function in newborn rhesus monkeys. Am. J. Respir. Crit. Care Med. 2001;164: 989-994.

17. Singh SP., Mishra NC, Rir-Sima-Ah J, Campen M, Kurup V, Razani-Boroujerdi S, et al. Maternal exposure to secondhand cigarette smoke primes the lung for induction of phosphodiesterase-4D5 isozyme and exacerbated Th2 responses: rolipram attenuates the airway hyperreactivity and muscarinic receptor expression but not lung inflammation and atopy. J. Immunol. 2009;183: 2115-2121.

18. Singh SP, Gundavarapu S, Peña-Philippides J, Rir-sima-ah J, Mishra N, Wilder J, et al. Prenatal secondhand cigarette smoke promotes Th2 polarization and impairs goblet cell differentiation and airway mucus formation. J. Immunol. 2011;187: 4542-4552.

19. Rehan VK, Asotra K, Torday JS. The effects of smoking on the developing lung: insights from a biologic model for lung development, homeostasis, and repair. Lung 2009;187: 281-289.

20. Vrijheid M, Casas M, Bergström A, Carmichael A, Cordier S, Eggesbø M, et al. European birth cohorts for environmental health research**.** Environ Health Perspect.; 2012; 120: 29-37.

21. Antonucci R, Contu P, Porcella A, Atzeni C, Chiappe S. Intrauterine smoke exposure: a new risk factor for bronchopulmonary dysplasia? J. Perinat. Med. 2004;32: 272-277.

22. De Luca G, Olivieri F, Melotti G, Aiello G, Lubrano L, Boner AL. Fetal and early postnatal life roots of asthma. J. Matern. Fetal Neonatal Med. 2010;23 Suppl 3: 80-83.

23. Singh SP, Barrett EG, Kalra R, Razani-Boroujerdi S, Langley RJ, Kurup V, et al. Prenatal cigarette smoke decreases lung cAMP and increases airway hyperresponsiveness. Am. J. Respir. Crit. Care Med. 2003;168: 342-347.

24. Fritzell JA Jr, Mao Q, Gundavarapu S, Pasquariello T, Aliotta JM, Ayala A, et al. Fate and effects of adult bone marrow cells in lungs of normoxic and hyperoxic newborn mice. Am J Respir Cell Mol Biol. 2009;40:575-587.

25.Wang SZ, Rosenberger CL, Espindola TM, Barrett EG, Tesfaigzi Y, Bice DE, et al. CCSP modulates airway dysfunction and host responses in an Ova-challenged mouse model. Am. J. Physiol. 2001;281: L1303-L1311.

26. Thurlbeck WM. Measurement of pulmonary emphysema. Am. Rev. Respir. Dis. 1967;95: 752-764.

27. Knudsen L, Weibel ER, Gundersen HJ, Weinstein FV, Ochs M. Assessment of air space size characteristics by intercept (chord) measurement: an accurate and efficient stereological approach. J Appl Physiol. 2010;108: 412-421.

28. Reynolds SD, Giangreco A, Power JH, Stripp BR. Neuroepithelial bodies of pulmonary airways serve as a reservoir of progenitor cells capable of epithelial regeneration. Am. J. Pathol. 2000;156: 269-278.

29. Rawlins EL, Ostrowski LE, Randell SH, Hogan BL. Lung development and repair: contribution of the ciliated lineage. Proc Natl Acad Sci U S A. 2007;104: 410-417.

30. Tompkins DH, Besnard V, Lange AW, Wert SE, Keiser AR, Smith AN, et al. Sox2 is required for maintenance and differentiation of bronchiolar Clara, ciliated, and goblet cells. PLoS One. 2009; 4:e8248.

31. Mishra NC, Rir-Sima-Ah J, Grotendorst GR, Langley RJ, Singh SP, Gundavarapu S, et al. Inhalation of sulfur mustard causes long-term T cell-dependent inflammation: Possible role of Th17 cells in chronic lung pathology. Int Immunopharmacol. 2012;13: 101-108.

32. Shi W, Bellusci S, Warburton D. Lung development and adult lung diseases. Chest 2007;132: 651-656.

33. Coalson JJ, Winter V, DeLemos RA. Decreased alveolarization in baboon survivors with bronchopulmonary dysplasia. Am. J. Respir. Crit. Care Med. 1995;152: 640-646.

34. Kwinta P, Pietrzyk JJ. Preterm birth and respiratory disease in later life. Expert Rev. Respir. Med. 2010;4: 593-604.

35. Narang, I. Review series: What goes around, comes around: childhood influences on later lung health? Long-term follow-up of infants with lung disease of prematurity. Chron. Respir. Dis. 2010;7: 259-269.

36. Benachi A, Delezoide AL, Chailley-Heu B, Preece M, Bourbon JR, Ryder T. Ultrastructural evaluation of lung maturation in a sheep model of diaphragmatic hernia and tracheal occlusion. Am. J. Respir. Cell Mol. Biol. 1999;20: 805-812.

37. Ahlfeld SK, Conway SJ. Aberrant signaling pathways of the lung mesenchyme and their contributions to the pathogenesis of bronchopulmonary dysplasia. Birth Defects Res. A Clin. Mol Teratol. 2012;94: 3-15.

38. D'Angio CT, Maniscalco WM. Bronchopulmonary dysplasia in preterm infants: pathophysiology and management strategies. Paediatr. Drugs 2004;6: 303-330.

39. Kinsella JP., Greenough A, Abman SH. Bronchopulmonary dysplasia. Lancet 2006;367**:** 1421-1431.

40. Reynolds S, Reynolds P, Pryhuber G, Finder J, Stripp B. Secretoglobins SCGB3A1 and SCGB3A2 define secretory cell subsets in mouse and human airways. Am. J. Respir. Crit. Care Med. 2002;166: 1498-1509.

41 . Kim C, Jackson E, Woolfenden A, Lawrence S, Babar I, Vogel S, et al. Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell 2005;121: 823-835.

42. Breier G. Functions of the VEGF/VEGF receptor system in the vascular system. Semin. Thromb. Hemost. 2000;26: 553-559.

43. McCullagh A, Rosenthal M, Wanner A, Hurtado A, Padley S, Bush A. The bronchial circulation--worth a closer look: a review of the relationship between the bronchial vasculature and airway inflammation. Pediatr. Pulmonol. 2010;45: 1-13.

44. Pusztaszeri MP, Seelentag W, Bosman FT. Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand Factor, and Fli-1 in normal human tissues. J. Histochem. Cytochem. 2006;54: 385-395.

45. Thébaud B, Ladha F, Michelakis ED, Sawicka M, Thurston G, Eaton F. Vascular endothelial growth factor gene therapy increases survival, promotes lung angiogenesis, and prevents

alveolar damage in hyperoxia-induced lung injury evidence that angiogenesis participates in alveolarization. Circulation 2005;112: 2477-2486.

46. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 1995;376: 66-70.

47. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML,et al. Failure of blood-island formation and vasculogenesis in flk-1-deficient mice. Nature 1995;376: 62-66.

48. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 1996;380: 435-439.

49. Ng YS, Rohan R, Sunday ME, Demello DE, D'Amore PA. Differential expression of VEGF isoforms in mouse during development and in the adult. Dev. Dyn. 2001;220: 112-121.

50. Ruhrberg C. Growing and shaping the vascular tree: multiple roles for VEGF. Bioessays 2003;25: 1052-1060.

51. Kalinichenko VV, Lim L, Stolz DB, Shin B, Rausa FM, Clark J, et al. Defects in pulmonary vasculature and perinatal lung hemorrhage in mice heterozygous null for the forkhead box f1 transcription factor. Dev. Biol. 2001;235: 489-506.

52. Rehan VK, Wang Y, Sugano S, Santos J, Patel S, Sakurai R, et al. In utero nicotine exposure alters fetal rat lung alveolar type II cell proliferation, differentiation, and metabolism. Am. J. Physiol. Lung Cell. Mol. Physiol. 2007;292: L323-L333.

53. Maritz GS, Harding R. Life-long programming implications of exposure to tobacco smoking and nicotine before and soon after birth: evidence for altered lung development. Int. J. Environ. Res. Public Health 2011;8: 875-898.

54. Perera F, Tang WY, Herbstman J, Tang D, Levin L, Miller R, et al. Relation of DNA methylation of 5'-CpG island of ACSL3 to transplacental exposure to airborne polycyclic aromatic hydrocarbons and childhood asthma. PLoS One. 2009;4: e4488.

55. Collins MH, Moessinger AC, Kleinerman J, Bassi J, Rosso P, Collins AM, et al. Fetal lung hypoplasia associated with maternal smoking: a morphometric analysis. Pediatr. Res. 1985;19: 408-412.

56. Knudsen L, Weibel ER, Gundersen HJ, Weinstein FV, Ochs M. Assessment of air space size characteristics by intercept (chord) measurement: an accurate and efficient stereological approach. J. Appl. Physiol. 2010;108: 412-421.

57. Jacob RE, Carson JP, Gideon KM, Amidan BG, Smith CL, Lee KM. Comparison of two quantitative methods of discerning airspace enlargement in smoke-exposed mice. Plos One 2009;4: e6670.

58. Srivastava PK, Dastidar SG, Ray A. Chronic obstructive pulmonary disease: role of matrix metalloproteases and future challenges of drug therapy. Expert Opin. Investig. Drugs 2007;16: 1069-1078.

59. Mocchegiani E, Giacconi R, Costarelli L. Metalloproteases/anti-metalloproteases imbalance in chronic obstructive pulmonary disease: genetic factors and treatment implications. Curr. Opin. Pulm. Med. 2011; 17 Suppl 1: S11-S19.

60. Belvisi MG, Bottomley KM. The role of matrix metalloproteinases (MMPs) in the pathophysiology of chronic obstructive pulmonary disease (COPD): a therapeutic role for inhibitors of MMPs? Inflamm. Res. 2003;52: 95-100.

61. Mokres LM, Parai K, Hilgendorff A, Ertsey R, Alvira CM, Rabinovitch M,et al. Prolonged mechanical ventilation with air induces apoptosis and causes failure of alveolar septation and angiogenesis in lungs of newborn mice. Am. J. Physiol. Lung Cell. Mol. Physiol. 2010;298: L23-L35.

62. Jobe AH, Ikegami M. Lung development and function in preterm infants in the surfactant treatment era. Annu. Rev. Physiol. 2000;62: 825-846.

63. Morrisey EE, Hogan BL. Preparing for the first breath: genetic and cellular mechanisms in lung development. Dev Cell. 2010;18: 8-23.

64. Hartling L, Wittmeier KD, Caldwell PH, Van der Lee JH, Klassen TP, Craig JC,et al. StaR Child Health group, StaR Child Health: developing evidence-based guidance for the design, conduct, and reporting of pediatric trials. Clin. Pharmacol. Ther. 2011;90: 727-731.

65. Albertine KH, Dahl MJ, Gonzales LW, Wang ZM, Metcalfe D, Hyde DM,et al. Chronic lung disease in preterm lambs: effect of daily vitamin A treatment on alveolarization. Am. J. Physiol. Lung Cell. Mol. Physiol. 2010;299: L59-L72.

66. D'Angio CT, Maniscalco WM. Bronchopulmonary dysplasia in preterm infants: pathophysiology and management strategies. Paediatr. Drugs 2004;6: 303-330.

67. Papaioannou A, Kostikas K, Kollia P, Gourgoulianis K. Review: Clinical implications for vascular endothelial growth factor in the lung: friend or foe? Respir. Res. 2006;7: 128-141.

68. Gebb SA, Shannon JM. Tissue interactions mediate early events in pulmonary vasculogenesis. Dev. Dyn. 2000;217: 159-169.

69. Baker CD, Balasubramaniam V, Mourani PM, Sontag MK, Black CP, Ryan B SL,et al. Cord blood angiogenic progenitor cells are decreased in bronchopulmonary dysplasia. Eur Respir J. 2012 Apr 10. [Epub ahead of print]

70. Suzuki M, Betsuyaku T, Nagai K, Fuke S, Nasuhara Y, Kaga K, et al. Decreased airway expression of vascular endothelial growth factor in cigarette smoke-induced emphysema in mice and COPD patients. Inhal. Toxicol. 2008;20: 349-359.

71. Tanigawa N, Amaya H, Matsumura M, Shimomatsuya T, Horiuchi T, Muraoka R, et al. Extent of tumor vascularization correlates with prognosis and hematogenous metastasis in gastric carcinomas. Cancer Res. 1996;56: 2671-2676.

72. Meller S, Bhandari V. VEGF levels in humans and animal models with RDS and BPD: temporal relationships. Exp Lung Res. 2011; 38:192-203.

73. Meller S, Bhandari V. VEGF levels in humans and animal models with RDS and BPD: temporal relationships. Exp Lung Res. 2011; 38:192-203.

74. Ramsay PL., DeMayo FJ, Hegemier SE, Wearden ME, Smith CV, Welty SE. Clara cell secretory protein oxidation and expression in premature infants who develop bronchopulmonary dysplasia. Am. J. Respir. Crit. Care Med. 2001;164: 155-161.

75. Abdel-Latif ME, Osborn DA. Intratracheal Clara cell secretory protein (CCSP) administration in preterm infants with or at risk of respiratory distress syndrome. Cochrane Database Syst. Rev. 2011;5: CD008308.

76. Pryhuber GS. Regulation and function of pulmonary surfactant protein B. Mol. Genet. Metab. 1998;64: 217-228.

77. Epaud R, Ikegami M, Whitsett JA, Jobe AH, Weaver TE, Akinbi HT. Surfactant protein B inhibits endotoxin-induced lung inflammation. Am. J. Respir. Cell. Mol. Biol. 2003;28: 373-378.

78. Moya F. Synthetic surfactants: where are we? Evidence from randomized, controlled clinical trials. J. Perinatol. 2009;Suppl. 2: S23-S28.

79. Londhe VA, Maisonet TM, Lopez B, Jeng JM, Xiao J, Li C, et al. Conditional deletion of epithelial IKKβ impairs alveolar formation through apoptosis and decreased VEGF expression

during early mouse lung morphogenesis. Respir. Res. 2011;12: 134. doi:10.1186/1465-9921-12-134.

80. Hoffmann D, Hoffmann I. Significance of exposure to sidestream tobacco smoke. IARC Sci. Publ. 1987;81: 3-10.

81. Schick S, Glantz S. Philip Morris toxicological experiments with fresh sidestream smoke: more toxic than mainstream smoke. Tob. Control 2005;14: 396-404.

82. Adgent MA, Environmental tobacco smoke and sudden infant death syndrome: a review. Birth Defects Res. B Dev. Reprod. Toxicol. 2006;77: 69-85.

83. Cooke JP, Ghebremariam YT. Endothelial nicotinic acetylcholine receptors and angiogenesis. Trends Cardiovasc. Med. 2008;18: 247-253.

84. Maouche K, Polette M, Jolly T, Medjber K, Cloëz-Tayarani I, Changeux JP, et al. {alpha}7 nicotinic acetylcholine receptor regulates airway epithelium differentiation by controlling basal cell proliferation. Am. J. Pathol. 2009;175: 1868-1882.

85. Mishra NC, Rir-Sima-Ah J, Langley RJ, Singh SP, Peña-Philippides JC, Koga T, et al. Nicotine primarily suppresses lung Th2 but not goblet cell and muscle cell responses to allergens. J. Immunol. 2008;180: 7655-7663.

86. Konishi H, Wu J, Cooke JP. Chronic exposure to nicotine impairs cholinergic angiogenesis. Vasc. Med. 2010;15: 47-54.

Figure Legends

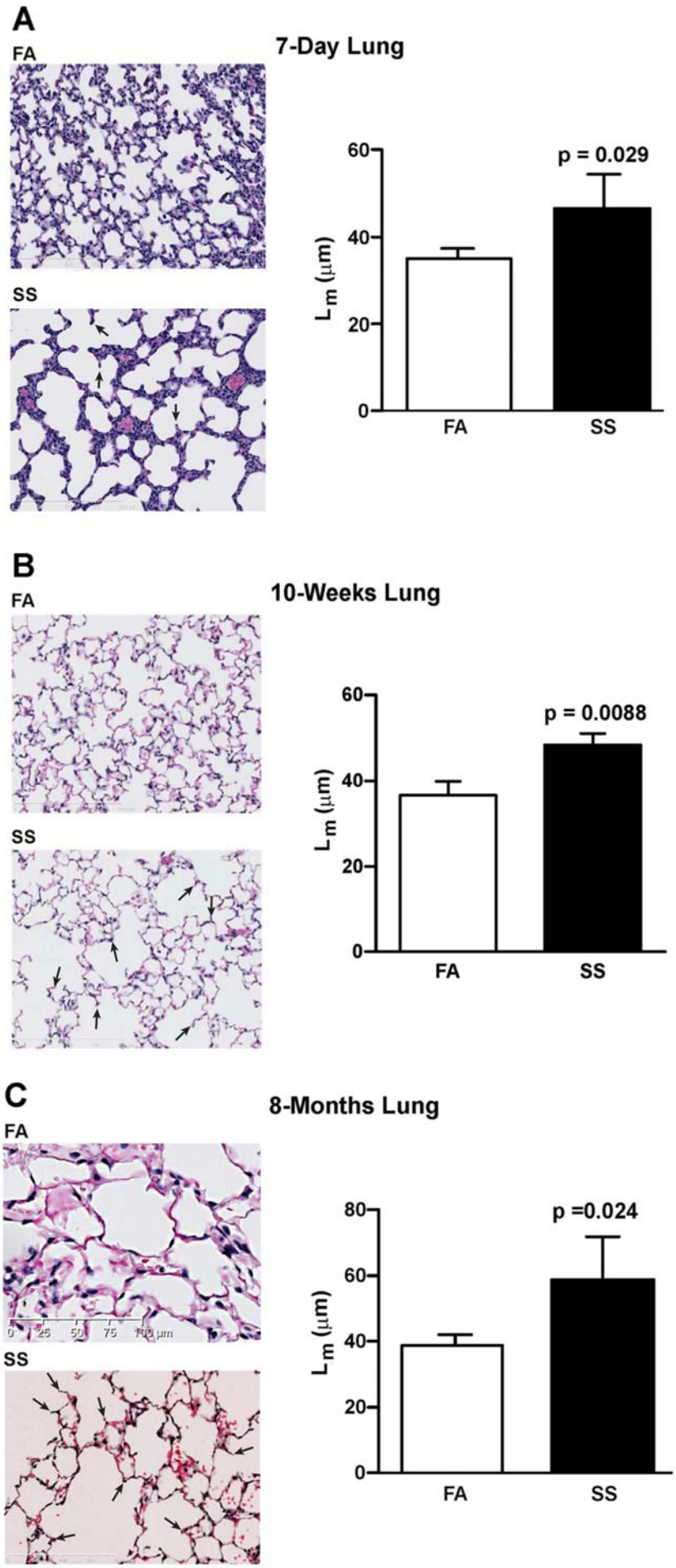
Fig. 1. Prenatal exposure to SS affects normal alveolar development. Representative sections (20X magnification) of lungs from 7-day old mice (**A**), 10-week old mice (**B**), and 8-month old mice (**C**). Right panels of the figures are the L_m measurements obtained by dividing the total length of a line drawn across the lung section (H&E stained) by the total number of intercepts encountered in 72 lines per lung section, an estimate of the size of the air-tissue interface. Arrows show blunted alveolar septal formation. FA = filtered air; SS = secondhand cigarette smoke; error bars are mean $\pm$ SD, n = 5

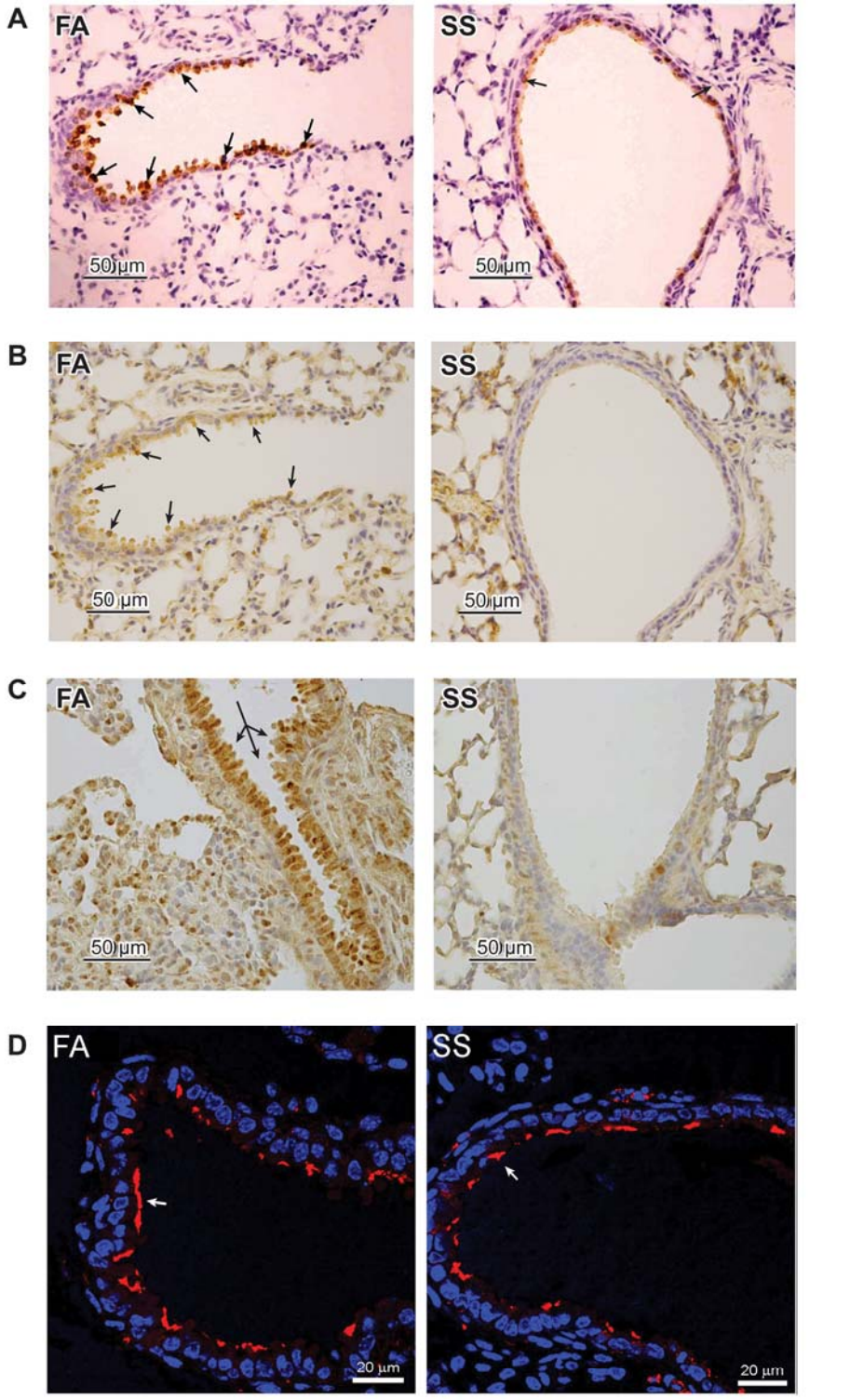
Fig. 2. Prenatal exposure to SS impairs the development of Clara and goblet cells but not ciliated cells. Lung sections of 7-day old mice were stained with anti-CCSP antibody (**A**), anti-SP-B antibody(**B**), anti-SPDEF antibody (GP954) (**C**), anti-β-tubulin antibody followed by Alexa 594-conjugated secondary antibody for ciliated cells (**D**), qPCR analysis for CCSP (**E_a**) and SP-B (**E_b**), and quantitation of surface cilia/μm basal lamia (**E_c**). The Lung airways area was examined at a magnification of 40x using a Nikon Eclipse E600W microscope. FA = filtered air (control); SS = secondhand cigarette smoke; arrows show stained cells in the airways; * p ≤0.05; ** p ≤0.01; NS = not significant; error bars are mean $\pm$ SD, n = 3-5 animals.

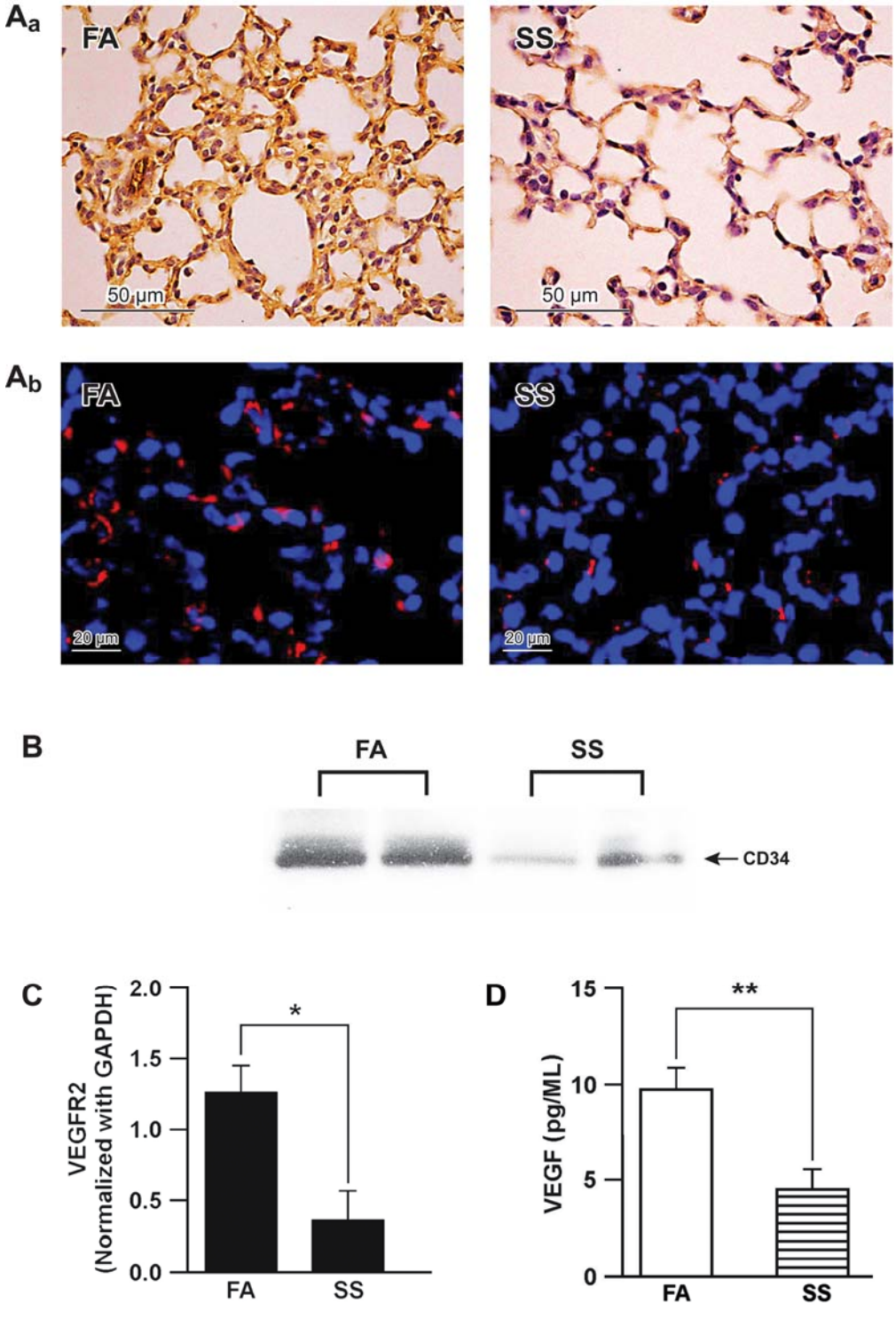
Fig. **3.** Prenatal exposure to SS inhibits angiogenesis. Representative lung (7-day old mice) IHC staining with an endothelial marker anti-CD34 antibody (40x magnification) (**A_a**), representative lung section immunofluorescence stained with anti-CD34 and anti-rat Alexa 555 (**A_b**), Western blot analysis of the lung homogenates (70 μg) from 7-day old mice using anti-CD34 antibody (**B**); VEGFR2 level by qPCR in the lungs of 7-day old mice(**C**), and VEGF level in BALF (10-week old mice) by ELISA (**D**). FA = filtered air (control); SS = prenatal secondhand cigarette smoke; * p ≤0.05**;** ** p ≤0.01; error bars are mean $\pm$ SD, n = 5.

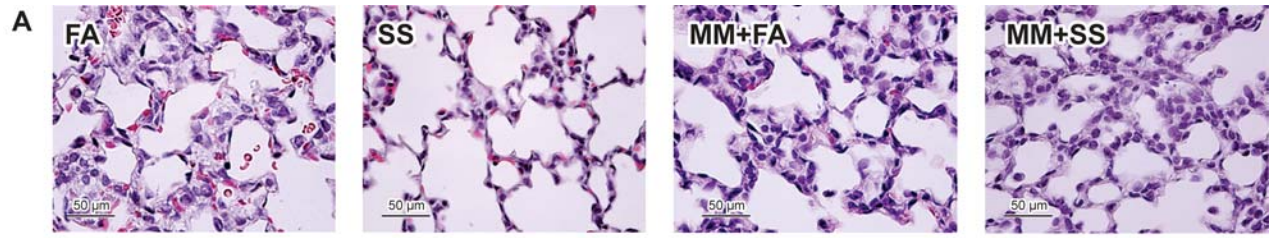
Fig. 4. Mecamylamine (MM) blocks gestational SS-induced effects on septation and angiogenesis. Representative (H&E) stained lung sections from 7-day old mice (40x magnification) (**A**), L_m values **(B**), IHC staining using anti-CD34 antibody (40x magnification) (**C_a**), immunofluorescence stained lung section using anti-CD34 and anti-rat Alexa 555 (**C_b**), and qPCR analysis of VEGFR2 from RNA from 7-day old lungs (**D**). FA = filtered air (control); SS = prenatal secondhand cigarette smoke, MM+FA = MM alone, and MM+SS = pups born to MM-treated breeder mice with gestational exposure to SS; ***** $p \leq 0.05$**;** ****** $p \leq 0.01$, and ******* $p \leq 0.001$; Error bar are mean $\pm$ SD, n = 5.

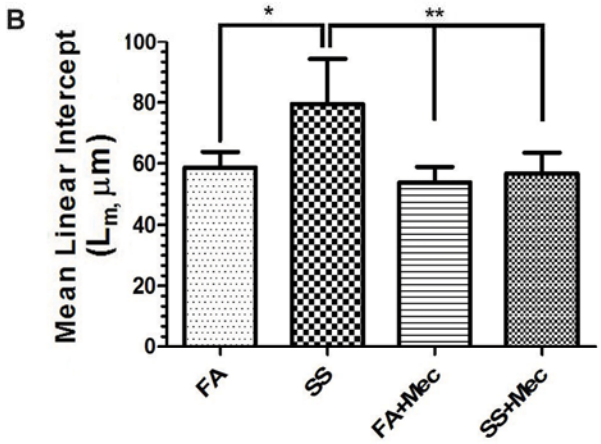
Fig. 5. Mecamylamine (MM) treatment blocks the effect of gestational SS on Clara cells numbers and function. qPCR analysis of RNA from 7-day old lung for SP-B (**A**) and CCSP (**B**). Representative lung sections IHC staining for SP-B (**C**) and CCSP (**D**) at 40x magnification. SP-B^+ cells/μm basal lamina (E_a) and $CCSP^+$ cells/μm basal lamina (E_b). FA = filtered air (control); SS = prenatal SS, and MM+SS = pups born to MM-treated breeder mice with gestational exposure to SS; ****** $p \leq 0.01$, and ******* $p \leq 0.001$; arrows indicate positively cells in the lung airways; Error bars are mean $\pm$ SD, n = 5.

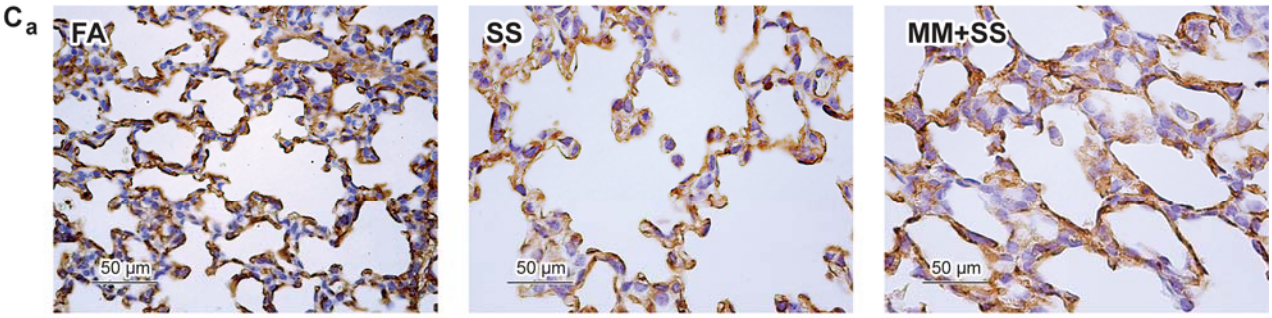

A
FA
7-Day Lung
SS
60
40
20
0
L_m (μm)
p = 0.029
FA
SS
B
FA
10-Weeks Lung
SS
60
40
20
0
L_m (μm)
p = 0.0088
FA
SS
C
FA
8-Months Lung
SS
80
60
40
20
0
L_m (μm)
p =0.024
FA
SS

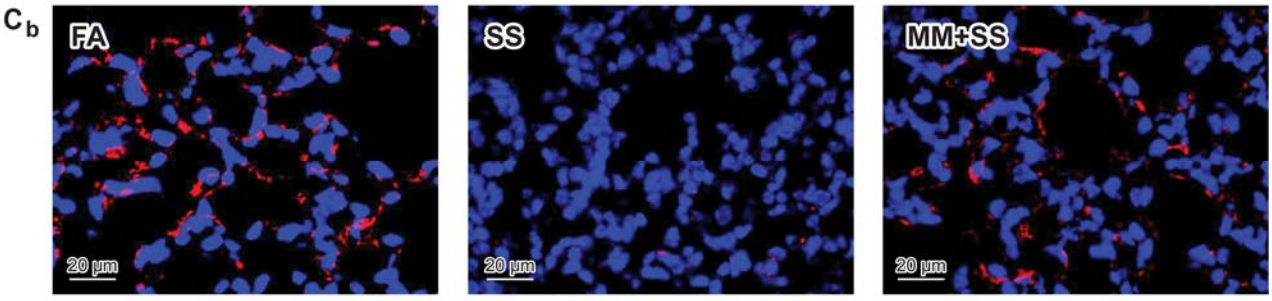

A
FA
SS
50 µm
50 µm
B
FA
SS
50 µm
50 µm
C
FA
SS
50 µm
50 µm
D
FA
SS
20 µm
20 µm
E
a
CCSP
(Normalized with GAPDH)
2.0
1.5
1.0
0.5
0.0
*
FA
SS
b
SP-B
(Normalized with GAPDH)
2.0
1.5
1.0
0.5
0.0
**
FA
SS
c
(surface cilia/µm lamina)
0.10
0.08
0.06
0.04
0.02
0.00
NS
FA
SS

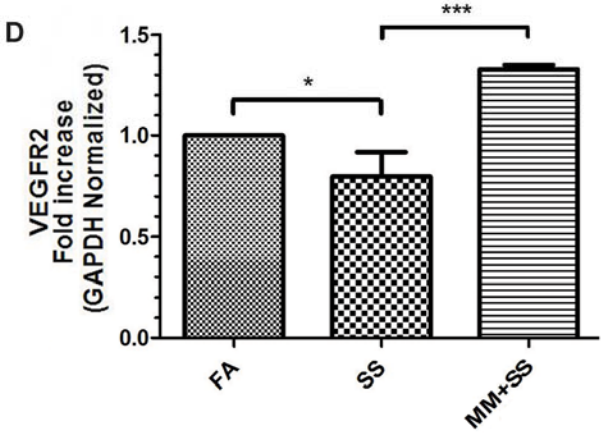

A a
FA
SS
50 μm
50 μm
A b
FA
SS
20 μm
20 μm
B
FA
SS
CD34
C
VEGFR2
(Normalized with GAPDH)
2.0
1.5
1.0
0.5
0.0
*
FA
SS
D
VEGF (pg/ML)
15
10
5
0
**
FA
SS

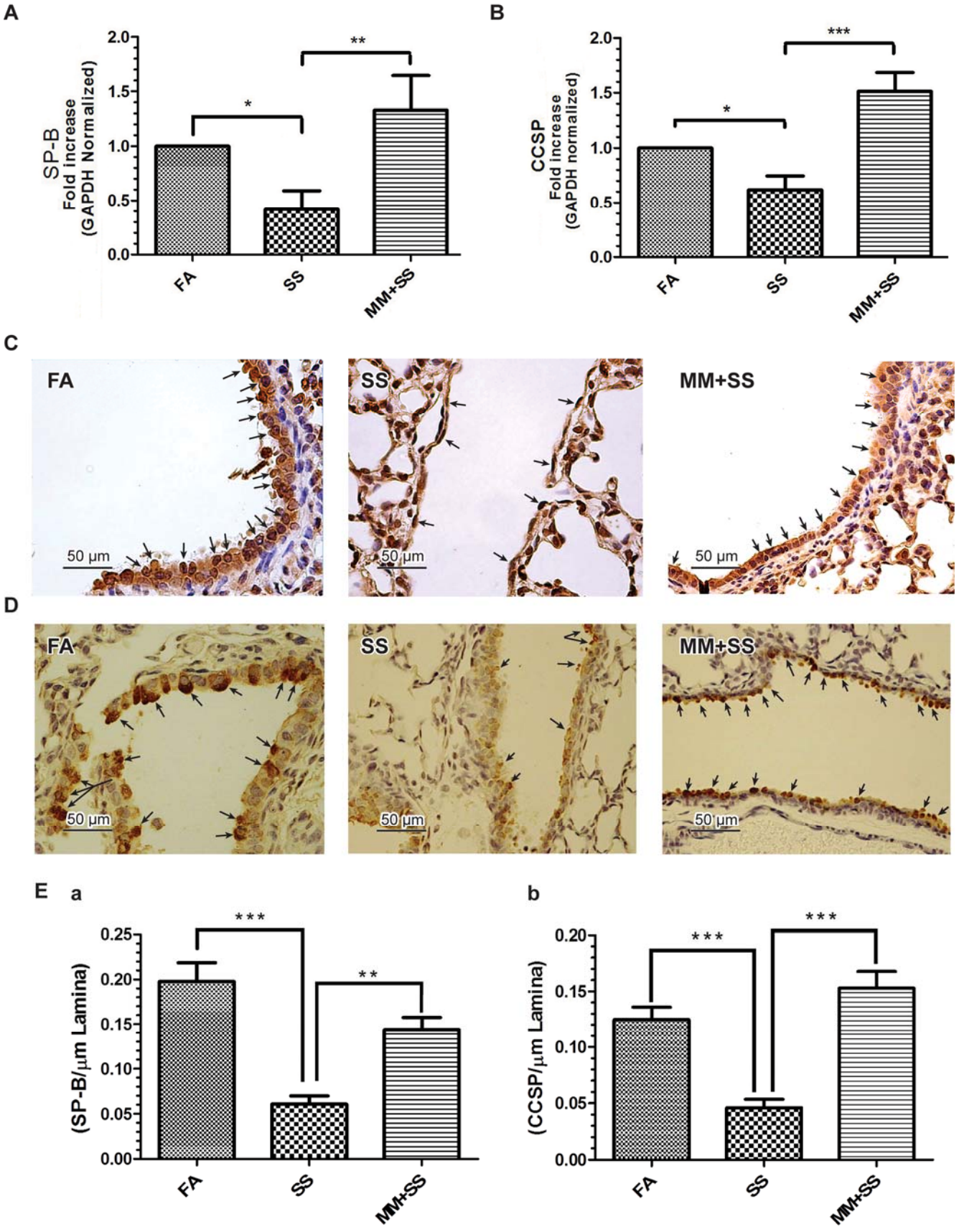

A
FA
SS
MM+FA
MM+SS
50 μm


B
Mean Linear Intercept
(L_m, μm)
100
80
60
40
20
0
*
**
FA
SS
FA+Mec
SS+Mec


Ca
FA
SS
MM+SS
50 μm


Cb
FA
SS
MM+SS
20 μm


D
VEGFR2
Fold Increase
(GAPDH Normalized)
1.5
1.0
0.5
0.0
*
***
FA
SS
MM+SS


A
SP-B
Fold Increase
(GAPDH Normalized)
2.0
1.5
1.0
0.5
0.0
*
**
FA
SS
MM+SS
B
CCSP
Fold increase
(GAPDH normalized)
2.0
1.5
1.0
0.5
0.0
*
***
FA
SS
MM+SS
C
FA
SS
MM+SS
50 µm
50 µm
50 µm
D
FA
SS
MM+SS
50 µm
50 µm
50 µm
E
a
(SP-B/µm Lamina)
0.25
0.20
0.15
0.10
0.05
0.00
***
**
FA
SS
MM+SS
b
(CCSP/µm Lamina)
0.20
0.15
0.10
0.05
0.00
***
***
FA
SS
MM+SS

"Supplemental"

Title: Gestational Exposure to Secondhand Cigarette Smoke Causes Bronchopulmonary Dysplasia Blocked by Mecamylamine

Running title: Gestational smoke induces bronchopulmonary dysplasia

Authors: Shashi P. Singh, Sravanthi Gundavarapu, Kevin R. Smith, Ali Imran Saeed, Richard J. Jaramillo, Neerad C. Mishra, Edward G. Barrett, Matloob Husain, Hitendra S. Chand, Kevin S. Harrod, Raymond J. Langley, and Mohan L Sopori

Corresponding Author:

Mohan Sopori, Ph.D.

Immunology Division, Lovelace Respiratory Research Institute

Albuquerque, NM 87108

Tel: (505) 348-9440

Fax: (505) 348-4986

Email: msopori@lrri.org

MATERIALS AND METHODS

Animals

Pathogen-free BALB/c mice (FCR Facility, Frederick, MD) were housed in shoebox-type plastic cages with hardwood chip bedding and conditioned to whole-body exposure in exposure chambers for 2 weeks before exposure to SS. The chamber temperature was maintained at 26 ± 2°C, and lights were set to a 12-hour on/off cycle. Food and water were provided ad libitum. All animal protocols were approved by the Institutional Animal Care and Use Committee.

Antibodies and reagents

Unless stated otherwise, all the reagents used in these experiments were purchased from Sigma Chemical Co. (St. Louis, MO).

Cigarette smoke generation and exposure

Mice were exposed to whole-body secondhand cigarette smoke (SS, the smoke released from the burning end of a cigarette) or fresh air (FA) for 6 hours/day, 7 days/week as described (E1). Briefly, 70-cm^3 puffs at the rate of 2/min were generated from 2R1 research cigarettes (Tobacco Health Research Institute, Lexington, KY) by a smoking machine (Type 1300; AMESA Electronics, Geneva, Switzerland). The smoke was captured from the lit end of the cigarette with a plastic manifold placed above it. Mice were placed in whole-body exposure chambers and exposed to either FA or SS (total particulate matter 1.52 ± 0.41 mg/m^3). It is a fairly low dose of SS that is approximately equivalent to the smoke exposure a pregnant woman would receive by sitting in a smoking bar for 3 hr/day throughout the gestational period (E2, E3) or equivalent to smoking <1/2 high tar/high nicotine cigarette/day during pregnancy (E1). Adult (3–4 month old) male and female mice were separately acclimatized either to SS or FA for 2 weeks, and then paired for mating under the same exposure conditions. After ensuring pregnancy by vaginal smear, pregnant mice were separated, housed singly in plastic cages, and continued to receive SS or FA until the

pups were born. Immediately after the birth of pups some mice from SS were placed in FA chamber (gestational SS-exposure only). Conversely, some mice from FA group were exposed to SS up to 10 wk after the birth (postnatal SS-exposure only). Mice were sacrificed at indicated times afterbirth.

Mecamylamine (MM) **treatment**

Breeder adult mice were subcutaneously implanted with miniosmotic pumps (ALZET model #; 2006, pumping rate: 0.15 µl/hr, ALZA Corporation, CA) containing saline (control) or MM (2.5 mg/ml in sterile saline), generating experimental groups FA, MM+ FA, and MM+ SS.

Bronchoalveolar lavage fluid (BALF) collection and VEGF analysis

Established protocols were followed to obtain BALF from the mice (E2). Briefly, 10-wk old FA (control) and SS-exposed were anesthetized and killed by exsanguination. Before excision of the lungs, the trachea was surgically exposed, cannulated and, while the left lung lobe was tied off with a silk thread, the right lobe was lavaged twice with 0.8 ml sterile Ca^{2+}/Mg^{2+}-free PBS (pH 7.4). Aliquots were pooled from individual animals and centrifuged to separate cells and BALF. The concentration of VEGF in BALF was determined by ELISA using mouse-specific VEGF ELISA kit (Biosource-Invitrogen, Camarillo, CA), according to the manufacturer's directions.

Tissue preparation, histochemistry, and immunostaining

Lungs harvested from 7 day old animals were inflated by tracheal instillation of formalin and kept in a formalin bath at a constant hydrostatic pressure of 20 cm for 24 h as described previously (E4). These lungs were washed in PBS (3x), dehydrated by series of ethanol washes, and embedded in paraffin (E4, E5). BALF from the lungs of 10-wk and 8-month old animals was obtained as described above; however, after removal of BAL, right lungs from some animals were frozen instantaneously in liquid N_2 and stored at -80 °C. When needed these lungs were used for making lung homogenates and total RNA.

H&E staining

Standard protocols were followed for H&E staining (E3). The slides were examined with a Nikon Eclipse E600W microscope with a digital camera (PXM1200F).

Morphometry

Mean linear intercept (L_m), a measure of interalveolar wall distance, was determined on H&E stained tissue sections using a Nanozoomer Digital Pathology (NDP) slide scanner (Hamamatsu K. K. Photonics, Hamamatsu City, Japan). L_m was obtained by dividing the NDP-generated total length of a line drawn across the lung section (scan resolution 20x) by the total number of intercepts encountered per lung section as described (E6, E7).

Immunohistochemical (IHC) staining for surfactant protein-B (SP-B)

For IHC staining lung section were deparaffinized by soaking in xylene for 10 min (2x), and then hydrated by 5-min treatment with decreasing concentrations of ethanol (100%, 95%, 70%, and 30%). Finally, the slides were rinsed three times with distilled water. The deparaffinized lung sections were immerged in 0.01 M sodium citrate buffer (pH 6.0) and heated in a microwave for 15 min at 90°C for antigen retrieval. Endogenous peroxidase activities were quenched with 3% H_2O_2 in methanol for 15 min at room temperature (RT). The tissue sections were blocked with 2% normal goat serum in PBS with 0.2% triton for 2 hours at RT, followed by overnight incubation at 4°C with rabbit proSP-B antibody (1:1000 dilution; Cat# AB3430; Chemicon, CA). Sections were then washed (6x for 5 min each) in PBS with 0.2% Triton and incubated for 30 min at RT with goat anti-rabbit (1:200 dilution; Cat# BA-1000; Vector, Burlingame, CA) for 1 hour at RT. Slides were then incubated in Vectastain Elite Immunoperoxidase system at RT for 30 min followed by 3,3'-Diaminobenzidine Peroxidase Substrate for 5 min at RT. Finally the sections were counterstained with hematoxylin, and analyzed with a Nikon Eclipse E600W microscope (PXM1200F).

IHC staining for Clara cells

Deparaffinized lung sections were stained for Clara cell secretory protein (CCSP, also called CC10, CC16, or uteroglobin) according to the method described by Reynolds et al. (E8). Briefly, lung sections (5 μm) were incubated with rabbit anti-CCSP antibody (1:50000 dilution; cat # 957, Vector, CA) overnight at 4°C. The sections were then washed as before and incubated with goat anti-rabbit antibody (Cat # BA-1000, Vector, CA) at a dilution of 1:200 for 1 hour at RT. After washing, the sections were incubated in a Vectastain Elite Immunoperoxidase system at RT for 30 min followed by 3,3'-Diaminobenzidine Peroxidase Substrate for 10 min at RT, and counterstained with hematoxylin, and examined microscopically.

Immunofluorescence staining of epithelial surface cilia

To visualize ciliated Clara cells, lung sections were treated with anti-β-tubulin antibody (E9, E10). Deparaffinized lung section were permeabilized with 0.2% Triton X-100 in PBS for 5 min and the sections were stained with rabbit anti-β-tubulin antibody (Abcam) for 2 hours followed by Alexa 594-conjugated anti-rabbit antibody (Invitrogen) for 1 hour. Sections were washed 5 times with wash buffer (PBS with 0.025% Triton X-100) and treated with 10 μg/ml Hoechst dye (Invitrogen). Washed sections were mounted in Mowiol Mounting Media (Sigma-Aldrich). All the above steps were performed at RT. Image fluorescence was visualized by confocal microscopy (Zeiss LSM510 META confocal microscope). Acquired images were processed in Photoshop CS3.

CD34 Immunostaining

To examine vascularization, lung sections were stained with anti-mouse CD34 antibody (1:2000 dilution, Cat#:119301, BioLegend, San Diego, CA) using standard procedures. CD34 staining produced minimal background staining. Slides were analyzed with a Nikon Eclipse E600W microscope with a digital camera (PXM1200F). Lung sections were also evaluated for CD34 expression by immunofluorescence. Briefly, deparaffinized, and hydrated lung sections were treated with 0.1M citrate buffer (pH 6.0).

Sections were then blocked and stained with anti-CD34 (BioLegend) and anti-rat Alexa 555 (Life Technologies, NY). Stained sections were mounted with 4', 6-diamidino-2-phenylindole (DAPI) containing Fluormount-GTM (Southern Biotech, Birmingham, AL) for nuclear staining. Fluorescently-labeled sections were analyzed using Axioplan 2 imaging system (Carl Zeiss Microimaging Inc., Thornwood, NY) and acquisition software Slidebook^{TM} 5 (Intelligent Imaging Innovations, Inc. Denver, CO).

Quantitation of IHC-stained SP-B^{+}, CCSP^{+}, and ciliated epithelial cells

The slides were scored for SP-B and CCSP-positive cells blind using the NDP system with Visiopharm software. Four randomly computer-selected areas of the each lung slide were quantified using approximately 7000 μm of airways basal lamina. A similar approach was used for the quantitation of surface cilia stained with anti β-tubulin antibody.

Trichrome Staining

To determine lung fibrosis, lung sections were stained with Masson's Trichrome (Sigma-Aldrich) to reveal collagen as described previously (E11).

Western blot analysis for CD34

CD34 were quantitated by Western blotting of the lung homogenates as described previously (E1, E3). Briefly, lung tissues were homogenized in RIPA buffer (20 mM Tris, 150 mM NaCl, 20 mM β-glyceryl-phosphate, 1% Triton-X, 10 mM NaF, 5 mM EDTA, 1 mM Na_3VO_4 and protease inhibitors (1 mM PMSF, and 1 μg/ml each of aprotinin, antipain, and leupeptin) at 4°C. Total protein content of the extracts was determined by the BCA Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer's directions. Lung homogenates proteins were resolved by SDS-PAGE on 10% precast gels (Invitrogen) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The blots were probed with anti-

CD34 antibody (MEC14.7, Abcam), and the antibody-bound proteins on the blot were detected with enhanced chemiluminescence (Amersham Biosciences, UK) on an X-ray film.

RNA isolation and quantitative PCR (qPCR)

Lung RNA was isolated as described previously (E2). The lung expression of SP-B, CCSP, VEGFR2, and GAPDH were determined by qPCR using the One-Step RT-PCR MasterMix containing TaqMan probes and a specific-labeled primer/probe sets (Applied Biosystems, Foster City, CA).

Data presentation and statistical analysis

Data were analyzed using Graph Pad Prism software 5.03 (Graphpad Software Inc., San Diego, CA). One-way ANOVA was used to compare the mean between the groups using the Tukey post-hoc test that compares all groups at 95% confidence intervals. The student's t test was used for comparison between two groups. Results are expressed as the mean $\pm$ SD. A p value of ≤ 0.05 was considered statistically significant.

References:

E1. Singh SP, Barrett EG, Kalra R, Razani-Boroujerdi S, Langley RJ, Kurup V, et al. Prenatal cigarette smoke decreases lung cAMP and increases airway hyperresponsiveness. Am. J. Respir. Crit. Care Med. 2003;168: 342-47.

E2. Singh SP, Mishra NC, Rir-Sima-Ah J, Campen M, Kurup V, Razani-Boroujerdi S, et al. Maternal exposure to secondhand cigarette smoke primes the lung for induction of phosphodiesterase-4D5 isozyme and exacerbated Th2 responses: rolipram attenuates the airway hyperreactivity and muscarinic receptor expression but not lung inflammation and atopy. J. Immunol. 2009;183: 2115-2121.

E3. Singh SP, Gundavarapu S, Peña-Philippides J, Rir-sima-ah J, Mishra N, Wilder J, et al. Prenatal secondhand cigarette smoke promotes Th2 polarization and impairs goblet cell differentiation and airway mucus formation. J. Immunol. 2011;187: 4542-4552.

E4. Fritzell JA Jr, Mao Q, Gundavarapu S, Pasquariello T, Aliotta JM, Ayala A, et al. Fate and effects of adult bone marrow cells in lungs of normoxic and hyperoxic newborn mice. Am J Respir Cell Mol Biol. 2009;40:575-587.

E5.Wang SZ, Rosenberger CL, Espindola TM, Barrett EG, Tesfaigzi Y, Bice DE, et al. CCSP modulates airway dysfunction and host responses in an Ova-challenged mouse model. Am. J. Physiol. 2001;281: L1303-L1311.

E6. Thurlbeck WM. Measurement of pulmonary emphysema. Am. Rev. Respir. Dis. 1967;95: 752-764.

E7. Knudsen L, Weibel ER, Gundersen HJ, Weinstein FV, Ochs M. Assessment of air space size characteristics by intercept (chord) measurement: an accurate and efficient stereological approach. J Appl Physiol. 2010;108:412-421.

E8. Reynolds SD, Giangreco A, Power JH, Stripp BR. Neuroepithelial bodies of pulmonary airways serve as a reservoir of progenitor cells capable of epithelial regeneration. Am. J. Pathol. 2000;156: 269-278.

E9. Rawlins EL, Ostrowski LE, Randell SH, Hogan BL. Lung development and repair: contribution of the ciliated lineage. Proc Natl Acad Sci U S A. 2007;104:410-417.

E10. Tompkins DH, Besnard V, Lange AW, Wert SE, Keiser AR, Smith AN, et al. Sox2 is required for maintenance and differentiation of bronchiolar Clara, ciliated, and goblet cells. PLoS One. 2009;4:e8248.

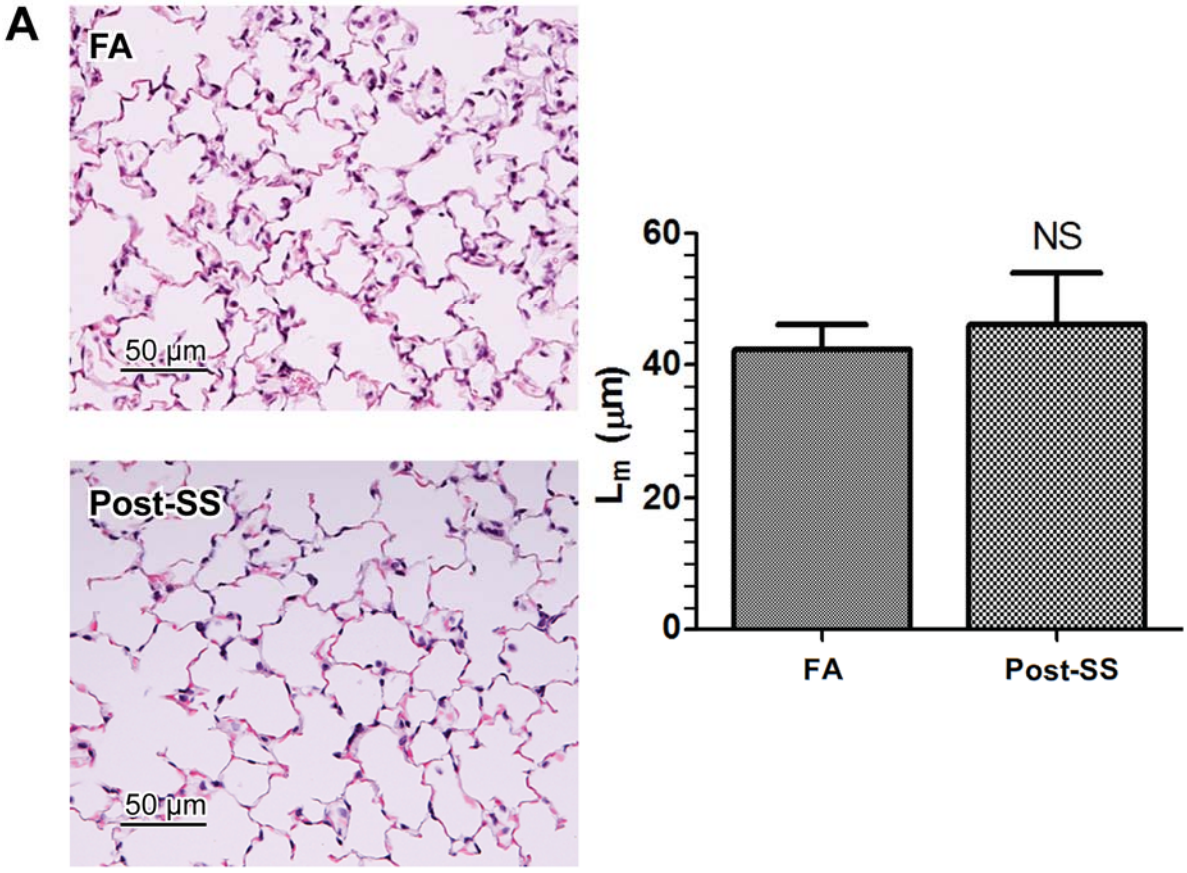
E11. Mishra NC, Rir-Sima-Ah J, Grotendorst GR, Langley RJ, Singh SP, Gundavarapu S, et al. Inhalation of sulfur mustard causes long-term T cell-dependent inflammation: Possible role of Th17 cells in chronic lung pathology. Int Immunopharmacol. 2012;13:101-108.

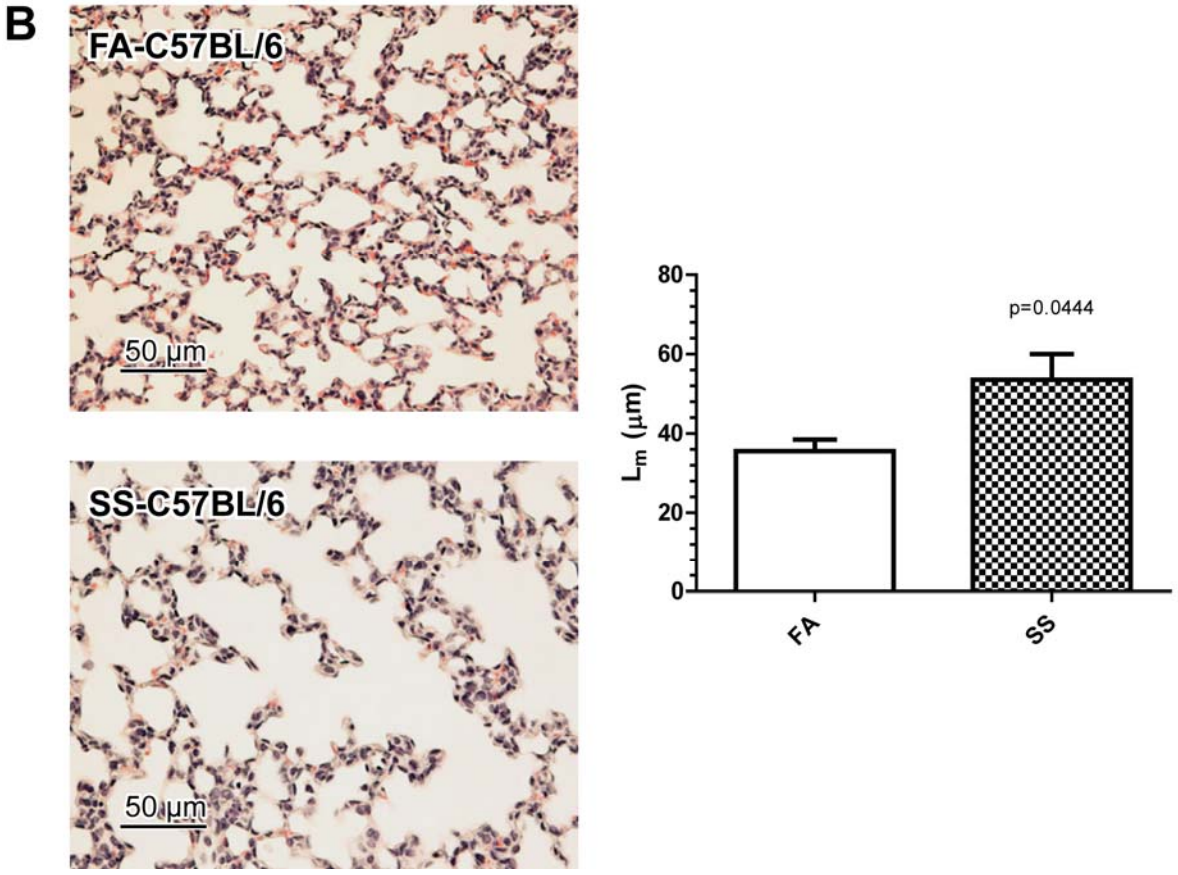
Supplemental Figure Legends

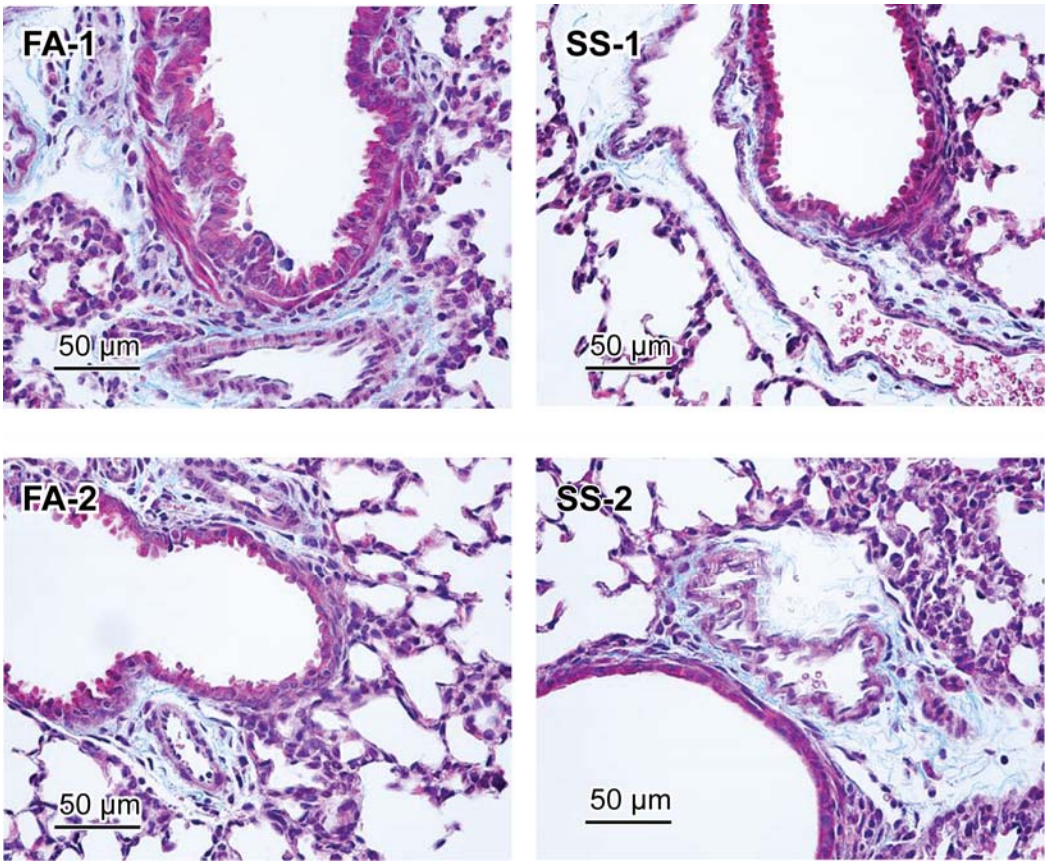
Fig. E1. (A) Postnatal exposure to SS does not affect alveolarization. Representative lung sections from 10-week postnatally FA (left, top) and postnatally SS-exposed (left, bottom) were stained with H&E and examined microscopically (40x magnification). L_m values were plotted (right bar graph)**. (B) C57BL/6 mice are also sensitive to gestational exposure to SS.** Representative lung sections (40x, H&E) from 7-day old C57BL/6 mice were examined. FA (left, top) and gestationally SS-exposed (left, bottom). L_m values were plotted (Fig. E1B, right bar graph). Values represent mean $\pm$ SD; NS = not significant; n = 3-5 mice/group).

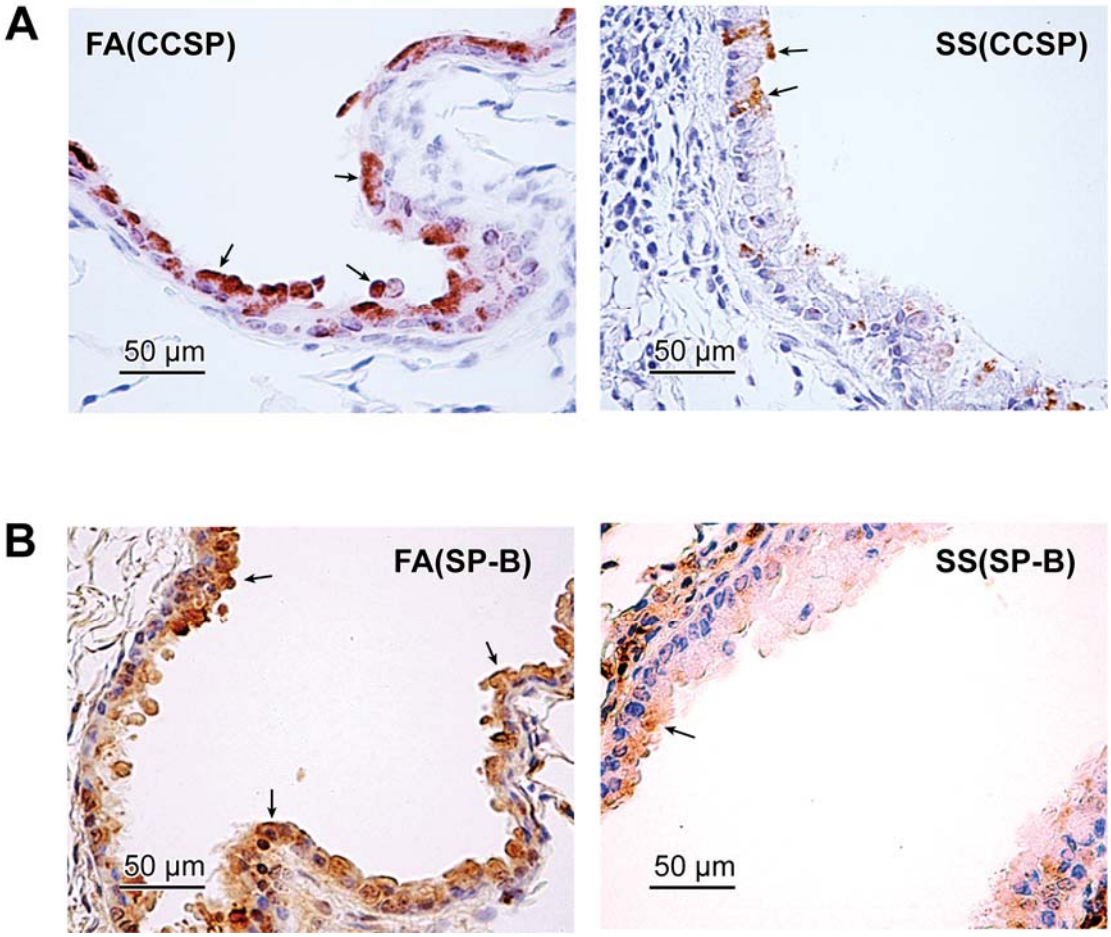
Fig. E2. Lung collagen content is unaffected by gestational SS. Lungs sections from 7-day old mice were stained with Masson's Trichrome (Sigma-Aldrich) to reveal collagen. FA (left panels) and SS (right panels). The blue staining represents collagen deposition (40x magnification).

Fig. E3. CCSP and SP-B are reduced in SS-exposed lungs at 10 wk afterbirth. Lung sections from 10-week old mice (gestationally exposed to either FA or SS) were stained with anti-CCSP antibody (A) or anti-SP-B antibody (B) as described in Materials and Methods. Representative lung sections (40x magnification) from 3 mice/group are shown.


A
FA
50 µm
Post-SS
50 µm
60
40
20
0
L_m (µm)
NS
FA
Post-SS


B
FA-C57BL/6
50 µm
SS-C57BL/6
50 µm
80
60
40
20
0
L_m (µm)
p=0.0444
FA
SS


FA-1
50 µm
SS-1
50 µm
FA-2
50 µm
SS-2
50 µm


A
FA(CCSP)
50 μm
SS(CCSP)
50 μm
B
FA(SP-B)
50 μm
SS(SP-B)
50 μm

Role of nicotinic receptors and acetylcholine in mucous cell metaplasia, hyperplasia, and airway mucus formation *in vitro* and *in vivo*

Sravanthi Gundavarapu, MS,[a] Julie A. Wilder, PhD,[a] Neerad C. Mishra, PhD,[a] Jules Rir-sima-ah, MS,[a] Raymond J. Langley, PhD,[a] Shashi P. Singh, PhD,[a] Ali Imran Saeed, MD,[b] Richard J. Jaramillo, BS,[a] Katherine M. Gott, BS,[a] Juan Carlos Peña-Philippides, BS,[a] Kevin S. Harrod, PhD,[a] J. Michael McIntosh, PhD,[c] Shilpa Buch, PhD,[d] and Mohan L. Sopori, PhD[a] *Albuquerque, NM, Salt Lake City, Utah, and Omaha, Neb*

Background: Airway mucus hypersecretion is a key pathophysiologic feature in a number of lung diseases. Cigarette smoke/nicotine and allergens are strong stimulators of airway mucus; however, the mechanism of mucus modulation is unclear.

Objectives: We sought to characterize the pathway by which cigarette smoke/nicotine regulates airway mucus and identify agents that decrease airway mucus.

Methods: IL-13 and γ-aminobutyric acid type A receptors ($GABA_AR$s) are implicated in airway mucus. We examined the role of IL-13 and $GABA_AR$s in nicotine-induced mucus formation in normal human bronchial epithelial (NHBE) and A549 cells and secondhand cigarette smoke–induced, ovalbumin-induced, or both mucus formation *in vivo*.

Results: Nicotine promotes mucus formation in NHBE cells; however, the nicotine-induced mucus formation is independent of IL-13 but sensitive to the $GABA_AR$ antagonist picrotoxin. Airway epithelial cells express α7-, α9-, and α10-nicotinic acetylcholine receptors (nAChRs), and specific inhibition or knockdown of α7- but not α9/α10-nAChRs abrogates mucus formation in response to nicotine and IL-13. Moreover, addition of acetylcholine or inhibition of its degradation increases mucus in NHBE cells. Nicotinic but not muscarinic receptor antagonists block allergen- or nicotine/cigarette smoke–induced airway mucus formation in NHBE cells, murine airways, or both.

Conclusions: Nicotine-induced airway mucus formation is independent of IL-13, and α7-nAChRs are critical in airway mucous cell metaplasia/hyperplasia and mucus production in response to various promucoid agents, including IL-13. In the absence of nicotine, acetylcholine might be the biological ligand for α7-nAChRs to trigger airway mucus formation. α7-nAChRs are downstream of IL-13 but upstream of $GABA_AR$α2 in the MUC5AC pathway. Acetylcholine and α7-nAChRs might serve as therapeutic targets to control airway mucus. (J Allergy Clin Immunol 2012;130:770-80.)

Key words: *Cigarette smoke, nicotine, nicotinic acetylcholine receptors, γ-aminobutyric acid receptors, acetylcholine, airway mucus*

Normal mammalian airway epithelium produces and is coated by mucins, such as MUC5B and MUC5AC, and after stimulation by an allergen/infection, MUC5AC is the predominant mucin produced in human airways. These mucins assist in clearing inhaled particulate matter from the airways.[1] However, excessive mucous cell metaplasia and mucus hypersecretion contribute to the pathology of many respiratory diseases, such as chronic obstructive pulmonary disease, asthma, and cystic fibrosis.[2] In addition, excessive mucus production prolongs lung infections and decreases lung function.[3] Cigarette smoke is a strong inducer of airway mucus production and a major risk factor for asthma, bronchitis, and chronic obstructive pulmonary disease.[4,5] Moreover, recent studies suggest that nicotine promotes airway mucus formation[6,7]; however, the mechanism by which cigarette smoke/nicotine promotes mucus formation is not well established. Studies have demonstrated that T_H2 cytokines, particularly IL-13, are key mediators of mucous cell metaplasia/hyperplasia and mucus production[8-10]; however, in a rat allergic asthma model, chronic nicotine treatment strongly downregulated IL-4 and IL-13 production but increased mucous cell metaplasia and mucus production in the lung.[11] Thus in this model of allergic asthma, nicotine might stimulate mucus formation independently or semi-independently of IL-13.

A number of nonneuronal cells, including T cells, macrophages, and lung epithelial cells, express nicotinic acetylcholine receptors (nAChRs) and might synthesize acetylcholine.[12,13] Mucus-producing lung epithelial cells from rats, mice, and human subjects also express several different γ-aminobutyric acid type A receptor ($GABA_AR$) subunits, which have been implicated in airway mucus formation.[14] Nicotine is a major constituent of cigarette smoke, and in the central nervous system nicotine activates $GABA_AR$s in some neurons. Moreover, the α7/α9/α10-nAChR antagonist methyllycaconitine (MLA) moderates mucus formation in monkey lungs.[6] We hypothesized that in airway epithelial cells nicotine activated $GABA_AR$s through nAChRs, thereby promoting mucus formation. In this communication we show that although normal human bronchial epithelial (NHBE) cells express the α7-, α9-, and α10-nAChR subunits, α7-nAChRs play a critical role in mucous cell metaplasia and mucus formation in NHBE

From [a]the Lovelace Respiratory Research Institute, Albuquerque; [b]Pulmonary and Critical Care Medicine, University of New Mexico, Albuquerque; [c]the Departments of Psychiatry and Biology, University of Utah, Salt Lake City; and [d]the Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha.

Supported in part by grants from the US Army Medical Research and Material Command (GW093005), the National Institutes of Health (R01-DA017003), and the Lovelace Respiratory Research Institute (IMMSPT).

Disclosure of potential conflict of interest: K. S. Harrod is a member of the Avisa Pharma Board of Directors. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication September 28, 2011; revised March 29, 2012; accepted for publication April 3, 2012.

Available online May 9, 2012.

Corresponding author: Mohan L. Sopori, PhD, Immunology Division, Lovelace Respiratory Research Institute, Albuquerque, NM 87108. E-mail: msopori@lrri.org.

0091-6749/$36.00

© 2012 American Academy of Allergy, Asthma & Immunology

doi:10.1016/j.jaci.2012.04.002

Abbreviations used
AB/PAS: Alcian blue/periodic acid–Schiff
ALI: Air-liquid interface
$GABA_AR$: γ-Aminobutyric acid type A receptor
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
IHC: Immunohistochemistry
MLA: Methyllycaconitine
MM: Mecamylamine
nAChR: Nicotinic acetylcholine receptor
NB: Neostigmine bromide
NHBE: Normal human bronchial epithelial
OVA: Ovalbumin
PIC: Picrotoxin
qPCR: Quantitative PCR
siRNA: Small interfering RNA
SS: Secondhand smoke
TBST: Tris-buffered saline containing 0.05% Tween-20
TCR: T-cell receptor

cells. Moreover, (1) nicotine promotes mucus formation independently of IL-13, (2) the normal biological ligand for the nAChRs in the bronchial epithelial cells for mucus formation might be acetylcholine, and (3) antagonists of nAChRs but not muscarinic receptors suppress mucus formation *in vivo* and *in vitro*.

METHODS

NHBE and A549 cells were cultured by using standard procedures.[15] DO11.10 ovalbumin (OVA)–T-cell receptor (TCR) transgenic mice on a BALB/c background were exposed to air, secondhand smoke (SS) or nicotine (1.5 mg total particulate material/m^3), heat-aggregated OVA aerosol (5 mg/m3), or OVA plus SS or nicotine for 2 weeks (6 h/d for 5 d/wk). In some experiments normal (wild-type) BALB/c mice were sensitized to *Aspergillus fumigatus* extract, as described previously.[16] Where indicated, mice were subcutaneously implanted with mecamylamine (MM)–containing miniosmotic pumps (2 mg/kg body weight per day) 3 weeks before exposure.[17] In another group of BALB/c mice, animals were first exposed subcutaneously to saline (control)– or MM-containing ALZET pumps (DURECT Corp, Cupertino, Calif) for 2 weeks and then sensitized with *A fumigatus* allergen extracts, as described in the Methods section in this article's Online Repository at www.jacionline.org. To determine the effects of nicotine, IL-13, or acetylcholine on NHBE or A549 cells, cells were treated with nicotine base (100 nmol/L), recombinant human IL-13 (10-50 ng/mL), or indicated concentrations of neostigmine bromide (NB), respectively, and the cultures were harvested approximately 48 hours later. $GABA_AR$, nAChR, or muscarinic receptor inhibitors were added at the indicated concentrations 2 hours before the addition of IL-13, nicotine, or NB. NHBE cells (5-μm-thick sections) were stained with Alcian blue/periodic acid–Schiff (AB/PAS) staining for mucus,[18,19] MUC5AC, or $GABA_AR\alpha2$ by using appropriate reagents, and the cell number and mucus volume were determined by using microscopy.[11,20] Murine lung sections were stained for MUC5AC and $GABA_AR\alpha2$ by using immunohistochemistry (IHC). Total RNA was isolated from lung tissues, NHBE cells, and A549 cells with TRI-Reagent (Molecular Research Center, Inc, Cincinnati, Ohio). $GABA_AR\alpha2$-specific mRNA was assayed by using SuperScript III One-Step RT-PCR with Platinum Taq (Invitrogen, Carlsbad, Calif). RT-PCR primers for $GABA_AR\alpha2$ were 5′-AGGCTTCCGTTATGATACAG (forward) and 5′-AGGACTGACCCCTAATACAG (reverse), and those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5′-CCCATCA CCATCTTCCAGGAG (forward) and 5′-TTCACCACCTTCTTCTTGATGT CAT (reverse). Quantitative PCR (qPCR) was performed with a TaqMan One-Step RT-PCR kit containing AmpliTaq Gold DNA polymerase with ABI primers and probes. Fold differences were determined by using the $2^{(-\Delta\Delta CT)}$ method.[21] nAChR subtypes were knocked down by specific small interfering RNAs (siRNAs); changes in specific mRNAs were determined 48 hours after siRNA treatment. Protein levels of $GABA_AR\alpha2$ were determined by means of Western blot analysis.[17] GraphPad Prism Software 5.03 (GraphPad Software, Inc, La Jolla, Calif) was used to determine statistical significance by means of 2-way ANOVA. Detailed methods are given in the Methods section in this article's Online Repository.

RESULTS

Nicotinic receptors are critical in mucus formation

We examined the effects of nicotine (100 nmol/L) on mucus formation in NHBE cells grown at the air-liquid interface (ALI). This is a realistic concentration of nicotine and is several-fold lower than the median effective concentration (10-100 μmol/L) of nicotine/nicotine agonists required to activate the ligand-gated cationic channel in neurons.[22] As seen with AB/PAS mucus staining (Fig 1, *A*), control NHBE cells have a low baseline level of mucus; however, when the cells were treated with nicotine, IL-13, or IL-13 plus nicotine for 48 hours (predetermined optimal time), the mucus content in these cells increased strongly. Furthermore, the α7/α9/α10-nAChR–specific antagonist MLA (Fig 1, *A*), as well as the nonselective nAChR antagonist MM (Fig 1, *B*), suppressed the nicotine plus IL-13–induced mucus formation in NHBE cells. MLA also blocked the increase in mucus formation in NHBE cells in response to either nicotine or IL-13 (see Fig E1 in this article's Online Repository at www.jacionline.org). To determine whether nicotine, IL-13, or both affected mucous cell hyperplasia and metaplasia, we measured the number of mucous cells per millimeter of basal lamina and the volume of mucus-containing cells (mucus volume per cubic millimeter of basement membrane), respectively. Nicotine and IL-13 significantly increased both mucous cell numbers (Fig 1, *C*, left panel) and volume (Fig 1, *C*, right panel), and these effects were blocked by MLA. These results suggest that both nicotine and IL-13 affect mucous cell physiology and require the activation of nAChRs (α7, α9/α10, or both). Although in some experiments a combined treatment with nicotine and IL-13 appeared to increase cell volume over that seen after individual treatment with IL-13 or nicotine, these differences varied from experiment to experiment and were not statistically significant (data not shown). Thus nicotine and IL-13 might affect the same downstream pathway or pathways for mucous cell hyperplasia/metaplasia and mucus production.

Nicotine and IL-13 increase MUC5AC expression

Mucin glycoproteins are the major constituents of airway mucus. *MUC5AC* is the dominant mucin gene expressed in airway goblet cells,[23] and IL-13 is known to increase MUC5AC expression in these cells.[24] NHBE cells were treated with nicotine, IL-13, or both and MUC5AC expression was examined by means of qPCR and IHC staining with the MUC5AC-specific antibody to ascertain whether nicotine, IL-13, or both also induced the expression of MUC5AC. Both nicotine and IL-13 significantly increased the mRNA expression of MUC5AC; however, combined treatment with nicotine and IL-13 did not cause significantly higher MUC5AC expression than that seen after nicotine or IL-13 alone, and pretreatment with MLA blocked the increase in MUC5AC mRNA caused by nicotine plus IL-13 (Fig 1, *D*). Similar results were observed by scoring for MUC5AC protein by using IHC staining (Fig 1, *E*).

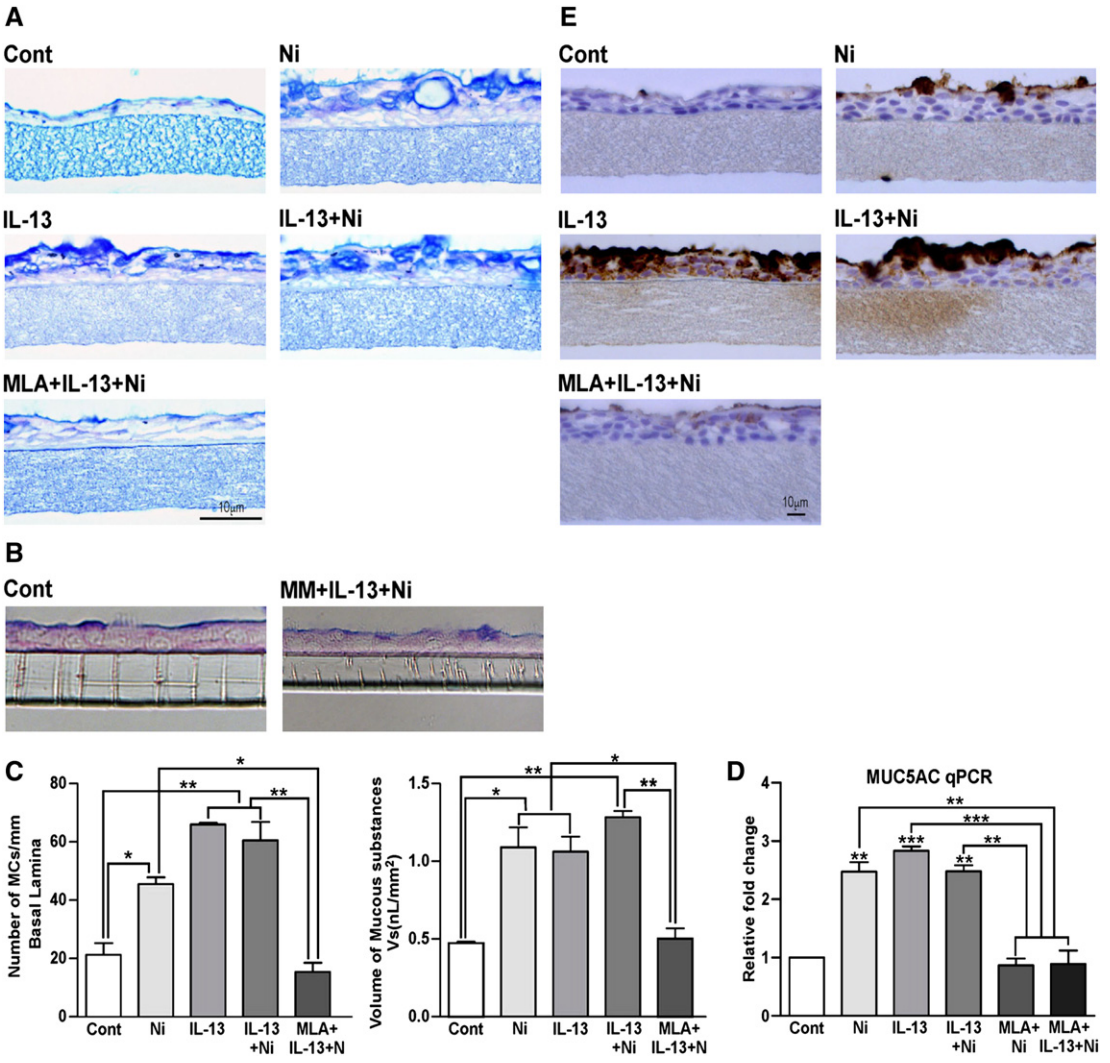


FIG 1. Nicotine *(Ni)* and IL-13 promote mucus formation in NHBE cells through nicotinic and $GABA_A$ receptors. In NHBE cells IL-13/nicotine–induced mucus is suppressed by 1 μmol/L MLA **(A)** and 1 μmol/L MM **(B)**. MLA blocks mucus-containing cells (**C**, *left panel*), mucous cell volume (Fig 1, *C, right panel*), MUC5AC mRNA **(D)**, and MUC5AC-positive cells **(E)**. Experiments were repeated at least 5 times, and *bars* represent means ± SEMs. $*P \leq .05$, $**P \leq .01$, and $***P \leq .001$.

Nicotine-induced MUC5AC is independent of IL-13

IL-13 is the critical cytokine in mucus formation and MUC5AC expression.[8-10,25] Although unlikely, it was possible that nicotine promoted MUC5AC/mucus formation by inducing IL-13 in NHBE cells. To ascertain this possibility, we determined IL-13 mRNA levels by using qPCR in NHBE cells before and after nicotine treatment. Unlike human Jurkat cells (positive control), qPCR analysis of NHBE cells (up to 40 cycles) did not show any detectable expression of IL-13 mRNA in the presence or absence of nicotine (see Fig E2 in this article's Online Repository at www.jacionline.org). Thus although both nicotine and IL-13 induce mucus formation and MUC5AC expression in NHBE cells, the nicotine-induced MUC5AC expression and mucus formation do not necessarily require IL-13.

α7-nAChRs are required for mucus formation

Neuronal nAChRs are pentameric structures, and in mammals nAChRs are derived from 8 α-subunit (α2-α7, α9, and α10) and 3 β-subunit (β2-β4) genes; however, α7 and α9 form functional homomeric receptors.[26] Moreover, α10 subunits are functional only in the presence of the α9 subunit[27]; the α7 and α10 subunits colocalize in rat sympathetic neurons.[28] Many nonneuronal cells, including T cells,[29] mast cells,[30] and macrophages, express nAChRs; mast cells express full-length α7-, α9-, and α10-nAChRs that respond interdependently to low concentrations of nicotine.[30] To ascertain whether a specific nAChR subtype mediated the effects of nicotine on mucus formation in NHBE cells, we determined the expression of nAChR subunits (α3, α4, α5, α7, α9, α10, and β4) in these cells. Examining the expression of α3-, α4-, and α7-nAChR subunits was warranted because they are expressed in the lung tracheal tissue,[31] and genome-wide association studies show single nucleotide polymorphisms in the gene cluster encoding α3/α5/β4-nAChR subunits in patients with lung cancer.[32] Our qPCR analysis suggested that the α3, α4, α5, and β4 subunits were essentially undetectable in NHBE cells (not shown); however, the cells expressed α7-, α9-, and α10-nAChR subunits (see Fig E3 in this article's Online

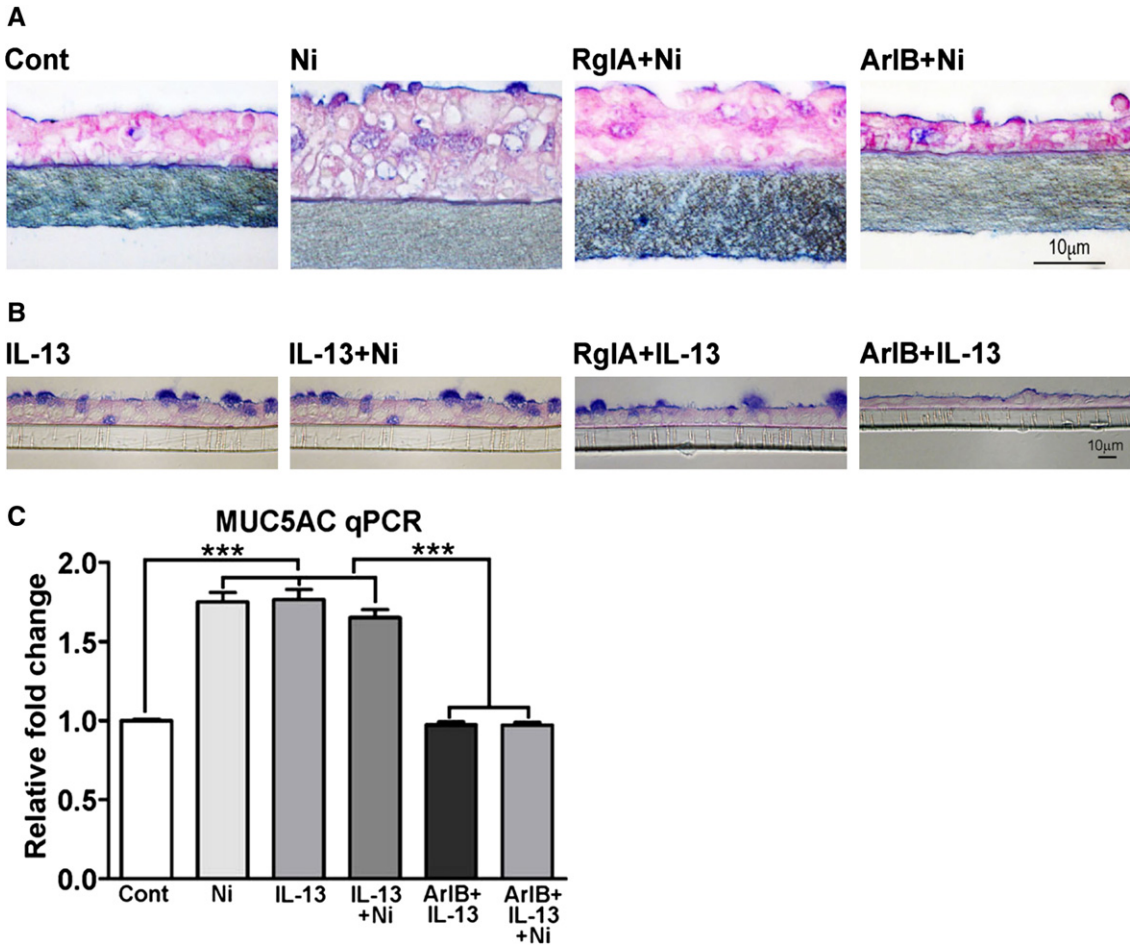


FIG 2. α7-nAChR–specific conotoxin peptides suppress nicotine *(Ni)*/IL-13–induced mucus in NHBE cells. Effects of RgIA (500 nmol/L) and ArIB[V11L, V16D] (500 nmol/L) on nicotine-induced **(A)** and IL-13–induced **(B)** mucous cell responses and on nicotine/IL-13–induced MUC5AC mRNA by using qPCR **(C)** are shown. Each experiment was repeated at least 3 times, and *bars* on MUC5AC are means ± SEMs from 3 inserts. ***$P \leq .001$.

Repository at www.jacionline.org). After normalizing with GAPDH, the mRNA expression of the α7 subunit was much higher than that of the α9 and α10 subunits (ie, α7 was detectable around 27 cycles and α9/α10 was detectable around 35 cycles of qPCR analysis). Thus NHBE cells express much higher levels of α7 than α9/α10 mRNA.

NHBE cells are generally refractory to various transfection/transduction approaches, and we were unable to knock down the expression of the α7-, α9-, or α10-nAChR subunits in these cells by using siRNA (not shown). Therefore to evaluate the role of α7-nAChR, α9/α10-nAChR, or both in mucus production, we used receptor subtype–specific conotoxin peptides to inhibit α7- and α9/α10-nAChRs in NHBE cells. At lower concentrations, the conotoxin peptides ArIB[V11L, V16D] and RgIA preferentially inhibit α7- and α9/α10-nAChRs, respectively.[33,34] By using these peptides at concentrations that showed minimum cross-inhibition, only ArIB[V11L, V16D] significantly reduced both nicotine-induced (Fig 2, *A*) and IL-13–induced (Fig 2, *B*) mucus production. Moreover, ArIB[V11L, V16D] (Fig 2, *C*), but not RgIA (not shown), also suppressed nicotine-induced expression, IL-13–induced expression, or both of MUC5AC mRNA, as determined by using qPCR. Thus α7-nAChRs are critical in the induction of airway mucus by nicotine and IL-13.

$GABA_A$Rs are downstream of α7-nAChRs

IL-13 has been shown to induce $GABA_A$Rs in the human bronchial epithelial cell line A549.[14] Although A549 cells do not produce mucus, they contain mRNAs for α7-, α9-, and α10-nAChRs (see Fig E4 in this article's Online Repository at www.jacionline.org), as well as α3-, α4-, and β2-nAChRs (not shown). Inhibiting $GABA_A$Rs with picrotoxin (PIC) also blocked mucus production in NHBE cells.[14] We used 3 approaches to determine whether the effects of nicotine on mucus formation involved $GABA_A$Rs.

First, we showed that the nonselective $GABA_A$R antagonist PIC inhibited the IL-13 plus nicotine–induced mucus (AB/PAS staining; Fig 3, *A*) and MUC5AC proteins (IHC staining; Fig 3, *B*) in NHBE cells or by IL-13 and nicotine individually (see Fig E1). Similarly, PIC also blocked the IL-13 plus nicotine–induced mucous cell hyperplasia (see Fig E5, *A*, in this article's Online Repository at www.jacionline.org), metaplasia (see Fig E5, *B*), and MUC5AC mRNA expression (see Fig E5, *C*) in NHBE cells.

Second, in NHBE cells nicotine, IL-13, or both induced the expression of $GABA_A$Rα2, as detected by using RT-PCR (Fig 3, *C*) and qPCR (Fig 3, *D*); the nicotine-induced expression of $GABA_A$Rα2 mRNA seen by RT-PCR was blocked by the α7/α9/α10-nAChR–specific antagonist MLA (Fig 3, *E*). Moreover,

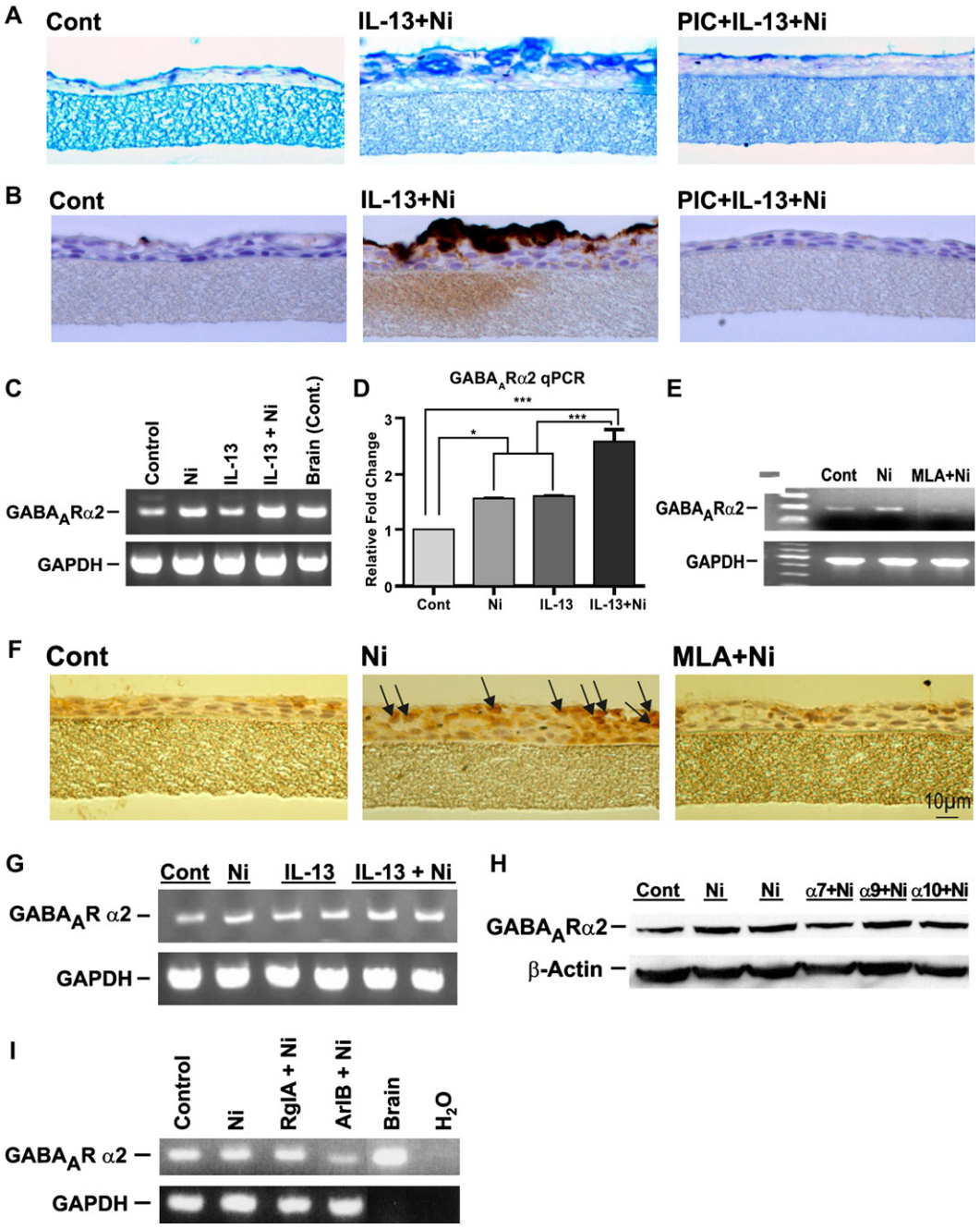


FIG 3. GABA$_A$Rs and α7-nAChRs are critical in nicotine *(Ni)*/IL-13–induced mucus formation. In NHBE cells effects of 50 μmol/L PIC on mucus-positive cells **(A)** and MUC5AC-positive cells **(B)** are shown. Nicotine/IL-13–induced GABA$_A$Rα2 expression is detected by using RT-PCR **(C)**, qPCR **(D)**, and IHC **(E)** and blocked by 1 μmol/L MLA **(F)**. In A-549 cells nicotine/IL-13–induced GABA$_A$Rα2 **(G)** is blocked by siRNA knockdown of α7-nAChRs **(H)** and α7-nAChR–specific conotoxin peptide ArIB[V11L, V16D] **(I)**. $*P \leq .05$ and $***P \leq .001$.

as assayed with IHC, MLA also blocked the nicotine-induced expression of GABA$_A$Rα2 in NHBE cells (Fig 3, *F*).

Third, A549 and NHBE cells express a number of GABA$_A$R subtypes. RT-PCR analysis suggested that both nicotine and IL-13 upregulated the expression of GABA$_A$Rα2 in A549 cells (Fig 3, *G*). Indeed, among several GABA$_A$R subtypes (GABA$_A$Rα2, GABA$_A$Rβ2, GABA$_A$Rγ, and GABA$_A$Rπ) only the expression of GABA$_A$Rα2 was consistently upregulated by IL-13 and nicotine in these cells (not shown). Moreover, MLA suppressed GABA$_A$Rα2 expression in A549 cells (see Fig E6 in this article's Online Repository at www.jacionline.org). Thus α7-nAChR, α9/α10-nAChRs, or both are involved in the increased expression of GABA$_A$Rα2 by nicotine in A549 cells.

To identify the type of nAChR subtype among the α7, α9, and α10 subunits that regulates GABA$_A$Rα2 expression and mucus formation, we used an siRNA approach to individually knock down α7-, α9-, and α10-nAChRs in A549 cells. Specific siRNA treatment selectively decreased the mRNA expression of α7-,

α9-, and α10-nAChRs by approximately 65%, 80%, and 70%, respectively (see Fig E7 in this article's Online Repository at www.jacionline.org). As seen by using Western blotting (Fig 3, *H*), the knockdown of α7, but not α9 and α10, significantly decreased nicotine-induced expression of $GABA_AR\alpha2$ in A549 cells. In these cells the nicotine-induced expression of $GABA_AR\alpha2$ mRNA was also blocked by the α7-specific ArIB[V11L, V16D] but not the α9/α10-specific RgIA conotoxin peptide (Fig 3, *I*). Because MLA blocked both nicotine- and IL-13–induced expression of $GABA_AR\alpha2$, activation of nAChRs must precede the activation of $GABA_AR\alpha2$ during mucus formation.

MM blocks $GABA_AR\alpha2$ and mucus production in mice

Cigarette smoke promotes goblet cell hyperplasia and mucus formation.[35] Similarly, sensitization with allergens, such as OVA, ragweed, and *Aspergillus* species extracts, promotes mucus formation in airways.[11,16] As seen by using immunostaining, a 2-week inhalation exposure of OVA-TCR transgenic mice on the BALB/c background to OVA, SS, or both strongly upregulated $GABA_AR\alpha2$ expression (Fig 4, *A*) and mucus formation (Fig 4, *B*) in the airways. However, when the animals were pretreated with the nAChR antagonist MM before exposure to SS plus OVA, the amplifying effects of SS plus OVA on $GABA_AR\alpha2$ expression (Fig 4, *A*) and airway mucus formation (Fig 4, *B*) were lost. Moreover, Western blot analysis of the lung extracts also indicated that MM blocked the OVA- and SS-induced expression of $GABA_AR\alpha2$ (Fig 4, *C*). MM by itself had no detectable effect on normal bronchial epithelium, such as gross histopathology or nuclear condensation (not shown). Similarly, qPCR analysis indicated that MM also blocked the SS-induced, OVA-induced, or both Muc5ac mRNA expression in the lung (Fig 4, *D*). In a separate experiment lungs from BALB/c mice exposed to air (control) or nicotine inhalation (1.5 mg/m3 for 6 h/d) for 2 weeks exhibited increased expression of $GABA_AR\alpha2$ protein by using Western blot analysis (Fig 4, *E*) and Muc5ac expression by using qPCR (Fig 4, *F*). Although an OVA-TCR transgenic murine model has been used extensively for delineating the mechanism of allergic asthma, a weakness of this model is that more than 90% of the peripheral T cells in these mice are directed to OVA and do not necessarily require sensitization with an allergen.[36] Therefore it is possible that the results might not be applicable to normal antigenic/allergic responses. To address this possibility, we used *A fumigatus* extracts containing the allergen in allergic bronchopulmonary aspergillosis[37] as the sensitizing allergen in normal BALB/c mice.[16] Results presented in Fig 5 suggest that, as in the OVA transgenic system, MM suppressed the mucus formation in response to *Aspergillus* species in normal BALB/c mice, including the increase in expression of Muc5ac (Fig 5, *A*), airway mucus formation by AB/PAS (Fig 5, *B*), and $GABA_AR\alpha2$ IHC staining (Fig 5, *C*). In addition, MM inhibited the inflammatory response and *Aspergillus* species–induced increase in eosinophil and lymphocyte counts, as determined by using BAL cell differential count (see Fig E8, *A*, in this article's Online Repository at www.jacionline.org), and the expression of the proinflammatory cytokines IL-13 (see Fig E8, *B*) and IFN-γ (see Fig E8, *C*) in the lung. Together these results suggest that nicotine promotes $GABA_AR\alpha2$ and mucus expression similar to that seen after exposure to cigarette smoke, and nAChRs play a critical role in both allergen- and cigarette smoke/nicotine–induced airway mucus formation *in vivo*.

Role of acetylcholine in mucus formation in NHBE cells

If the activation of nAChRs on airway epithelial cells were to play a decisive role in mucus formation, even in the absence of nicotine/cigarette smoke (eg, nonsmokers), it is important to understand how mucus-promoting molecules, such as allergens, would activate nAChRs *in vivo*. Nicotine is not normally found in mammals; rather, nicotinic cholinergic transmission is mediated by the neurotransmitter acetylcholine.[38] There is increasing evidence that airway epithelial cells have the enzymes to synthesize, degrade, and transport acetylcholine.[12,39] qPCR analysis indicated that NHBE cells express primarily M3 and lower levels of M1 and M2 muscarinic receptors (see Fig E9, *A*, in this article's Online Repository at www.jacionline.org). Acetylcholine is a highly labile molecule and difficult to assay. Therefore to ascertain that acetylcholine promotes mucus formation in airway epithelial cells, we determined first whether inhibiting the degradation of acetylcholine through the acetylcholinesterase inhibitor NB promoted mucus formation in NHBE cells. NHBE cells were treated with indicated concentrations of NB in the absence of IL-13 or nicotine. As little as 5 μmol/L NB (Fig 6, *A*) significantly upregulated the mucus formation in NHBE cells. NB also increased MUC5AC mRNA to less impressive but highly statistically significant level (Fig 6, *B*). Second, acetylcholine (100 μmol/L) was added to NHBE cells, and 48 hours later, the cells were analyzed for MUC5AC and $GABA_AR\alpha2$ mRNA expression by using qPCR. Compared with control cells, addition of acetylcholine significantly increased the expression of both MUC5AC and $GABA_AR\alpha2$ by approximately 2-fold (see Fig E9, *B* and *C*). These results suggest that acetylcholine is a trigger for mucus formation in airway epithelial cells. However, it should be noted that we were unable to detect acetylcholine in NHBE cells in the presence of NB and IL-13. It is likely that the cellular level of acetylcholine in these cells is lower than the sensitivity of our HPLC assay.

Acetylcholine is the biological ligand for both nicotinic and muscarinic receptors. NHBE cells were treated with the nonselective muscarinic receptor antagonist atropine before NB or IL-13 to ascertain that acetylcholine mediates its promucoid effects through nAChRs. Fig 6, *C*, shows that atropine had no significant effect on the MUC5AC mRNA expression induced by NB, IL-13, or NB plus IL-13. Similarly, atropine did not affect mucus formation (AB/PAS staining) in NHBE cells in response to NB or IL-13 (Fig 6, *D*). Moreover, unlike MLA, the increased level of $GABA_AR\alpha2$ in NHBE cells in response to nicotine or IL-13 plus nicotine was not affected by atropine treatment (Fig 6, *E*). These results suggest that, in the absence of nicotine or nicotine-containing substances, acetylcholine might be the critical molecule in the activation of nAChRs for mucus production in NHBE cells.

DISCUSSION

NAChRs are seen in tissues from both vertebrates and invertebrates, such as nematodes and insects.[40] In the central nervous system nAChRs are ligand-gated ion channels that mediate fast synaptic cholinergic transmission, and these properties are strongly conserved across species. nAChRs are present in neuronal[32] and many nonneuronal[13,29,30] cell types. At least in some

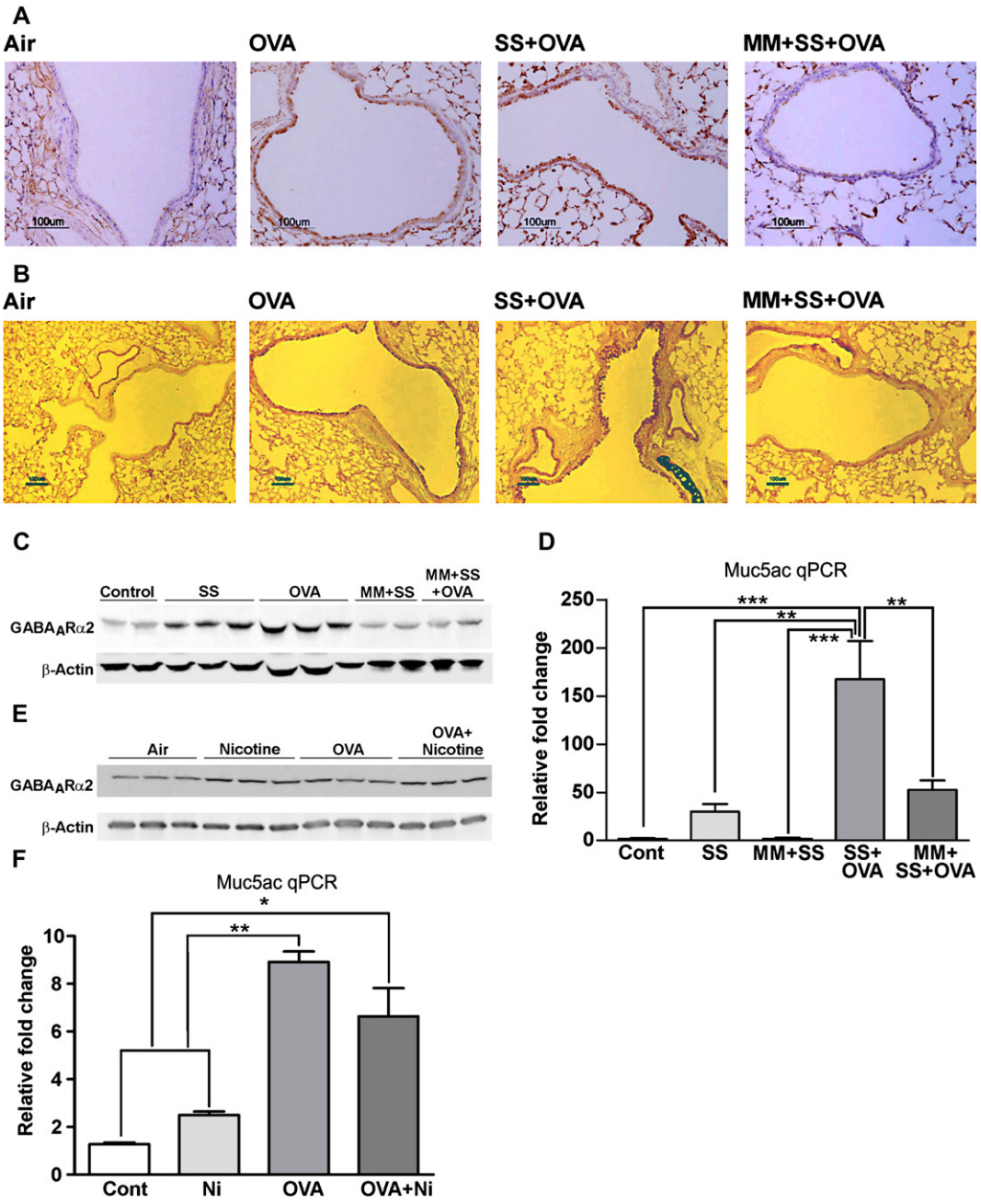


FIG 4. MM blocks OVA/SS/nicotine *(Ni)*–induced airway mucus, Muc5ac, and GABA$_A$Rα2 expression in OVA-transgenic mice. Mice received indicated treatments, and lungs were tested for GABA$_A$Rα2 by using IHC **(A)**, mucus by using AB/PAS **(B)**, GABA$_A$Rα2 by using Western blots of lung extracts **(C)**, levels of Muc5ac by using qPCR **(D)**, GABA$_A$Rα2 by using Western blots of lung extracts **(E)**, and levels of Muc5ac by using qPCR **(F)**. The results represent 2 independent experiments (3-6 mice per group). $*P \leq .05$, $**P \leq .01$, and $***P \leq .001$.

nonneuronal cell types nAChRs are not ligand-gated ion channels but signal through second messengers.[29] Thus far, the function of nAChRs in nonneuronal cells is described primarily as regulatory[41]; however, the near-ubiquitous presence of nAChRs in various cell types and their evolutionary conservation suggest that these receptors might have some critical functions outside the central nervous system.[42] Results presented herein indicate that nAChRs are essential for airway mucus production, as well as mucous cell hyperplasia and metaplasia, in response to nicotine and allergen *in vivo*, *in vitro*, or both, and it is likely that nAChRs are critical in other cellular functions.

Nicotine is immunosuppressive and anti-inflammatory,[41] and our previous *in vivo* experiments indicated that chronic low-dose nicotine exposure significantly inhibited some parameters of allergic asthma, including a dramatic reduction in levels of T_H2 cytokines/chemokines, such as IL-13, IL-4, IL-5, and eotaxin, and atopy, yet the nicotine-treated rats exhibited increased mucous cell metaplasia and mucus formation in the airways.[11] Possible

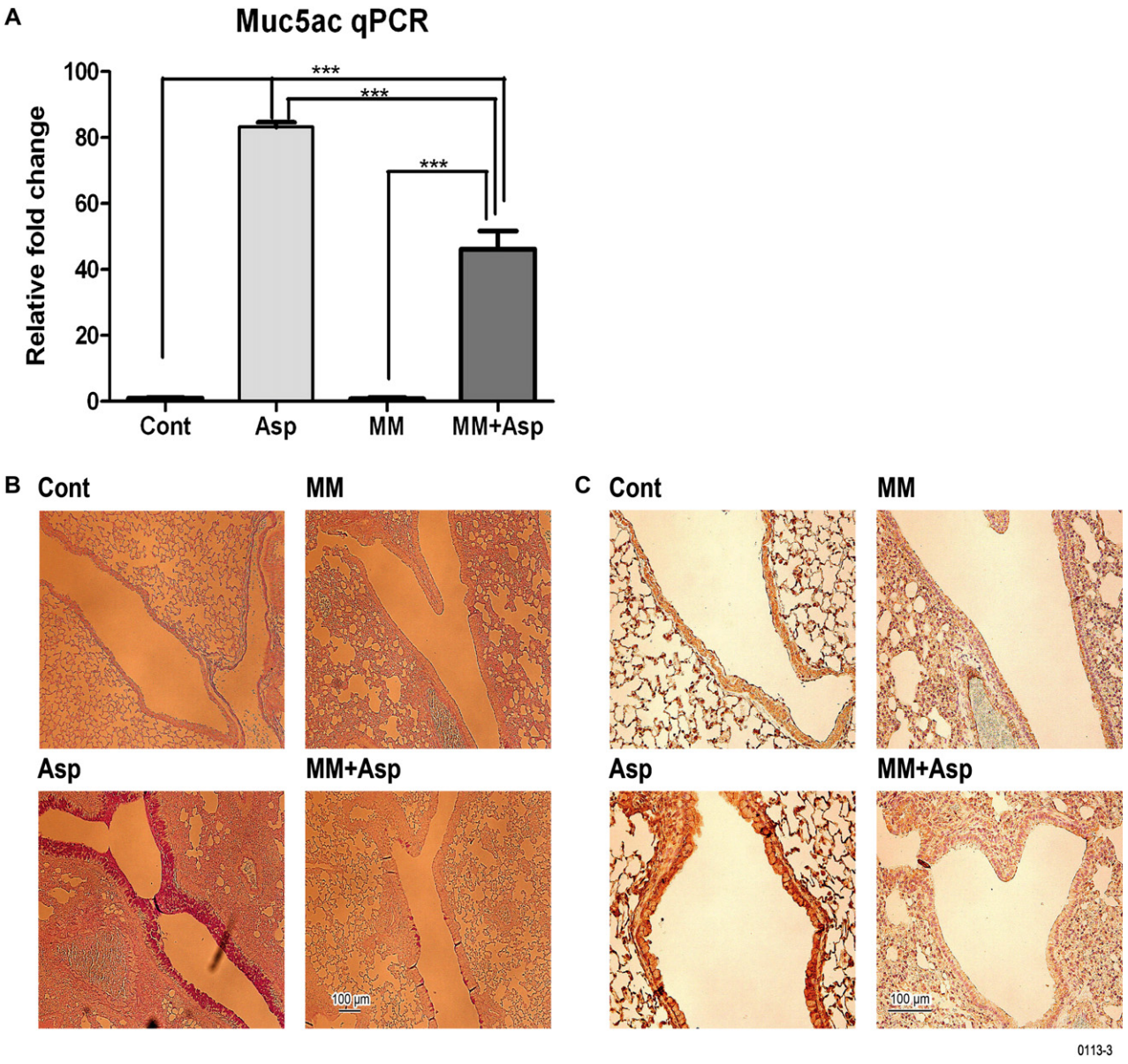


FIG 5. *Aspergillus* species induces MM-sensitive $GABA_AR\alpha2$ expression and mucus formation in the lung. BALB/c mice were sensitized with *Aspergillus fumigatus (Asp)* extracts and, where indicated, received MM. Lungs were tested for Muc5ac by using qPCR **(A)**, mucus by using AB/PAS staining **(B)**, and $GABA_AR\alpha2$ by using IHC **(C)**. The results represent 2 independent experiments with 3 to 6 mice per group. $***P \leq .001$.

explanations for these paradoxical results are that nicotine either decreased the amount of IL-13 required or substituted for IL-13 in mucus production. Our results clearly indicate that nicotine acts independently of IL-13 in promoting mucus formation and mucous cell metaplasia/hyperplasia. The ability of nAChR inhibitors to block nicotine- and IL-13–induced mucus production suggests that both IL-13 and nicotine activate nAChRs to trigger mucus formation, and IL-13's effects are upstream of nAChRs.

Previous studies have shown that IL-13 affects mucus by increasing $GABA_AR$ expression in NHBE cells.[14] We showed that $GABA_AR$ activation is downstream of nAChR activation in mucus formation and MUC5AC expression, and of the known $GABA_AR$ subtypes expressed in NHBE cells, $GABA_AR\alpha2$ was the only one that was significantly upregulated by IL-13 and nicotine in NHBE cells. $GABA_AR\alpha2$ was also the only $GABA_AR$ subtype the expression of which was increased by OVA, secondhand cigarette smoke, or both in OVA-TCR transgenic BALB/c mice. The interaction between nAChRs and $GABA_AR$s has been shown in the central nervous system,[43] and in *Caenorhabditis elegans* cholinergic motor neurons activate GABAergic neurons.[44] Moreover, rhesus monkeys exposed prenatally to nicotine show increased GABA signaling in the lungs[6]; however, the significance of this observation is not clear because prenatal nicotine exposure also affects the development of several organs, including the lung.[45] Thus although the mechanism by which nicotine promotes the GABAergic response has not been fully delineated, it is clear that $GABA_AR\alpha2$ is critical in nicotine- and IL-13–mediated mucus formation.

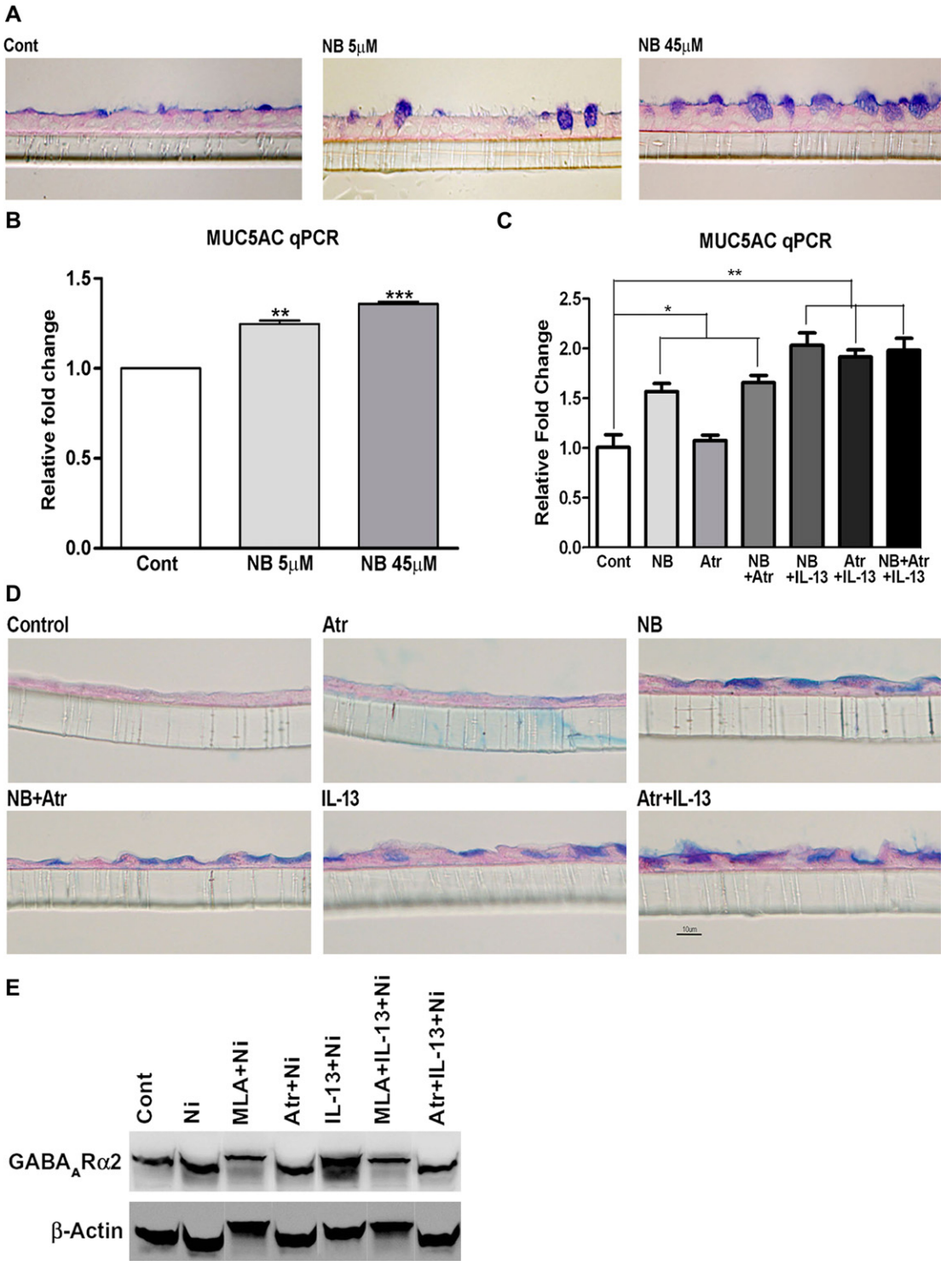


FIG 6. NB promotes mucus formation in NHBE cells. NHBE cells were treated with indicated concentrations of NB or 1 μmol/L atropine *(Atr)* and scored for NB-induced mucus **(A)** and MUC5AC **(B)**. Atropine's effects on NB/IL-13–induced MUC5AC **(C)**, mucus **(D)**, and GABAARα2 protein expression **(E)** are shown. The experiment in Fig 6, *A*, *B*, and *E*, was repeated twice. *Bars* represent means ± SEMs from 3 separate inserts. *Ni*, Nicotine. $*P \leq .05$, $**P \leq .01$, and $***P \leq .001$.

To ascertain the role of nAChRs in the regulation of airway mucus *in vivo*, we used 2 models of allergic asthma to trigger mucus formation. OVA-TCR transgenic BALB/c mice (a frequently used model for lung allergic responses) were exposed to OVA, SS, or both. These treatments promoted airway inflammation (leukocytic infiltration in the lung), airway mucus formation, and increased expression of Muc5ac and GABA$_A$Rα2 in the lung. However, when the animals were treated with the nonselective

nAChR antagonist MM, the inflammatory and mucoid responses to OVA, SS, or both were significantly reduced, suggesting that nAChRs are intimately involved in the regulation of allergen-induced inflammation and mucus formation. The major question about the suitability of using OVA-TCR transgenic animals to study the regulation of allergic asthma is that although normal naive (unimmunized) animals have extremely low frequencies of antigen-specific T cells,[46] the majority of the peripheral T cells in the OVA transgenic animals are directed to OVA and do not require immunization to detect OVA-induced T-cell proliferation,[36] although in the absence of presensitization by OVA, the cells might not differentiate into T_H2-type cells. Therefore we used normal (wild-type) BALB/c mice and the allergen–*A fumigatus* extracts that require sensitization to induce airway responses[16] and cause allergic bronchopulmonary aspergillosis in human subjects.[47] In these animals MM was able to ameliorate *Aspergillus* species–induced airway inflammation and various indices of airway mucoid response. Thus activation of nAChRs is critical in allergen-induced mucous cell metaplasia and airway mucus formation.

Recently, MLA was shown to suppress mucus formation in monkey lungs.[6] Although MLA was believed to preferentially block α7-nAChRs, recent reports suggest that MLA also reacts with α9/α10-nAChRs.[48] NHBE cells express α7-, α9- and α10-nAChRs, and α10-nAChRs are functional only in association with α9 subunits[27]; moreover, the α7 and α9 subunits colocalize in rat sympathetic neurons.[28] In rat mast cells the suppressive effect of nicotine on leukotriene production is mediated by an interdependent action of α7-, α9- and α10-nAChRs.[30] Therefore to identify the nAChR subtype or subtypes that regulated mucus formation, we used specific conotoxin peptides to inhibit α9/α10- and α7-nAChRs and observed that only the α7-specific conotoxin peptide ArIB[V11L, V16D][33,34] blocked the nicotine- and IL-13–induced mucus production in NHBE cells. This inference was further confirmed by the demonstration that knockdown of α7- but not α9-/α10-specific mRNA blocked the nicotine- and IL-13–induced expression of $GABA_AR\alpha2$ in A549 cells.

Although these results clearly implicate α7-nAChRs in mucous cell physiology and mucus production, it was important to understand how the activation of these receptors would be regulated in nonsmokers (ie, in the absence of nicotine). *In vivo* cholinergic transmission involves both nicotinic and muscarinic receptors and is mediated by acetylcholine. There is increasing evidence that many nonneuronal cells, including airway epithelial cells, express enzymes to synthesize, degrade, and transport acetylcholine.[12,39] Indeed, blocking the degradation of acetylcholine by the cholinesterase inhibitor NB promoted mucus formation and increased MUC5AC expression in NHBE cells in the complete absence of IL-13 or nicotine. Acetylcholine is the biological ligand for both nAChRs and muscarinic receptors, and bronchial epithelial cells have functional muscarinic receptors.[49] Results with MLA and atropine suggest that muscarinic receptors are not involved in the IL-13– or NB (acetylcholine)–induced mucus formation seen in bronchial epithelial cells. Although, with the use of nAChR inhibitors, we were able to show that the effects of IL-13 on mucus formation in NHBE cells are regulated by nAChRs, we were unable to show that IL-13 induces detectable levels of acetylcholine in these cells. Nonetheless, it is likely that in the absence of nicotine, acetylcholine is important in airway mucus formation and mucous cell hyperplasia/metaplasia. Together, our results suggest that α7-nAChRs, $GABA_AR\alpha2$, and the acetylcholine metabolic pathway or pathways can serve as potential targets to control airway mucus formation. A tentative scheme by which nAChRs might regulate airway mucus is presented in Fig 7.

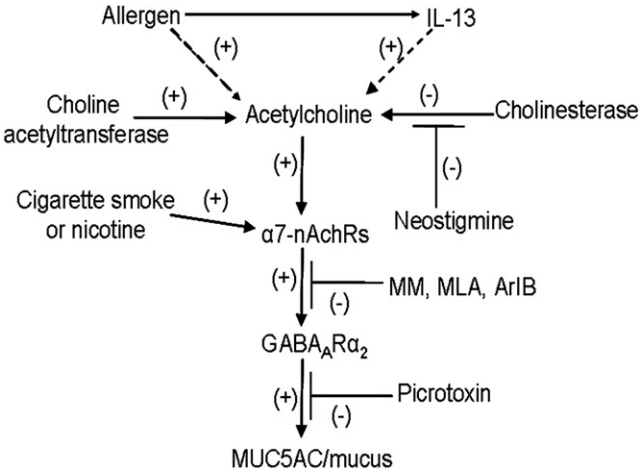


FIG 7. Potential relationship between nAChRs and mucus formation in NHBE cells. Allergens or IL-13 directly or indirectly increase acetylcholine levels in airway epithelial cells. Acetylcholine activates α7-nAChRs that increase $GABA_AR\alpha2$ expression that is blocked by nAChR antagonists (MM, MLA, and ArIB[V11L, V16D]). Increased $GABA_AR\alpha2$ stimulates mucus formation that is blocked by the $GABA_AR$ antagonist PIC. *Dashed lines* represent not formally proved interactions.

Clinical implications: This study shows that nicotine and acetylcholine promote mucus formation independently of IL-13 and in a manner totally dependent on the activation of α7-nAChRs. Moreover, nicotinic receptor antagonists block mucus formation.

REFERENCES

1. Hovenberg HW, Davies JR, Carlstedt I. Different mucins are produced by the surface epithelium and the submucosa in human trachea: identification of MUC5AC as a major mucin from the goblet cells. Biochem J 1996;318:319-24.
2. Turner J, Jones CE. Regulation of mucin expression in respiratory diseases. Biochem Soc Trans 2009;37:877-81.
3. Vestbo J. Epidemiological studies in mucus hypersecretion. Novartis Found Symp 2002;248:3-19, 277-82.
4. Schuster EA. Environment needs nurses who care. As I see it. Am Nurse 1992;24:25.
5. Markewitz BA, Owens MW, Payne DK. The pathogenesis of chronic obstructive pulmonary disease. Am J Med Sci 1999;318:74-8.
6. Fu XW, Wood K, Spindel ER. Prenatal nicotine exposure increases GABA signaling and mucin expression in airway epithelium. Am J Respir Cell Mol Biol 2011;44:222-9.
7. Gundavarapu S, Wilder JA, Rir-Sima-Ah J, Mishra NC, Singh SP, Jaramillo R, et al. Modulation of mucous cell metaplasia by cigarette smoke/nicotine through GABAA receptor expression [abstract]. Am J Respir Crit Care Med 2011;181:A5442.
8. Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. Science 1998;282:2261-3.
9. Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, et al. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. Nat Med 2002;8:885-9.
10. Wills-Karp M. Interleukin-13 in asthma pathogenesis. Immunol Rev 2004;202:175-90.
11. Mishra NC, Rir-Sima-Ah J, Langley RJ, Singh SP, Pena-Philippides JC, Koga T, et al. Nicotine primarily suppresses lung Th2 but not goblet cell and muscle cell responses to allergens. J Immunol 2008;180:7655-63.

12. Proskocil BJ, Sekhon HS, Jia Y, Savchenko V, Blakely RD, Lindstrom J, et al. Acetylcholine is an autocrine or paracrine hormone synthesized and secreted by airway bronchial epithelial cells. Endocrinology 2004;145:2498-506.
13. Fujii T, Takada-Takatori Y, Kawashima K. Basic and clinical aspects of non-neuronal acetylcholine: expression of an independent, non-neuronal cholinergic system in lymphocytes and its clinical significance in immunotherapy. J Pharmacol Sci 2008;106:186-92.
14. Xiang YY, Wang S, Liu M, Hirota JA, Li J, Ju W, et al. A GABAergic system in airway epithelium is essential for mucus overproduction in asthma. Nat Med 2007; 13:862-7.
15. Atherton HC, Jones G, Danahay H. IL-13-induced changes in the goblet cell density of human bronchial epithelial cell cultures: MAP kinase and phosphatidylinositol 3-kinase regulation. Am J Physiol Lung Cell Mol Physiol 2003;285:L730-9.
16. Singh SP, Gundavarapu S, Pena-Philippides JC, Rir-Sima-Ah J, Mishra NC, Wilder JA, et al. Prenatal secondhand cigarette smoke promotes th2 polarization and impairs goblet cell differentiation and airway mucus formation. J Immunol 2011;187: 4542-52.
17. Singh SP, Kalra R, Puttfarcken P, Kozak A, Tesfaigzi J, Sopori ML. Acute and chronic nicotine exposures modulate the immune system through different pathways. Toxicol Appl Pharmacol 2000;164:65-72.
18. Singh SP, Mishra NC, Rir-Sima-Ah J, Campen M, Kurup V, Razani-Boroujerdi S, et al. Maternal exposure to secondhand cigarette smoke primes the lung for induction of phosphodiesterase-4D5 isozyme and exacerbated Th2 responses: rolipram attenuates the airway hyperreactivity and muscarinic receptor expression but not lung inflammation and atopy. J Immunol 2009;183:2115-21.
19. El-Zimaity HM, Ota H, Scott S, Killen DE, Graham DY. A new triple stain for Helicobacter pylori suitable for the autostainer: carbol fuchsin/Alcian blue/hematoxylin-eosin. Arch Pathol Lab Med 1998;122:732-6.
20. Harkema JR, Hotchkiss JA. In vivo effects of endotoxin on intraepithelial mucosubstances in rat pulmonary airways. Quantitative histochemistry. Am J Pathol 1992;141:307-17.
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001;25:402-8.
22. Fucile S, Sucapane A, Eusebi F. Ca2+ permeability of nicotinic acetylcholine receptors from rat dorsal root ganglion neurones. J Physiol 2005;565:219-28.
23. Fahy JV. Goblet cell and mucin gene abnormalities in asthma. Chest 2002;122: 320S-6S.
24. Curran DR, Cohn L. Advances in mucous cell metaplasia: a plug for mucus as a therapeutic focus in chronic airway disease. Am J Respir Cell Mol Biol 2010; 42:268-75.
25. Izuhara K, Ohta S, Shiraishi H, Suzuki S, Taniguchi K, Toda S, et al. The mechanism of mucus production in bronchial asthma. Curr Med Chem 2009;16:2867-75.
26. Boyd RT. The molecular biology of neuronal nicotinic acetylcholine receptors. Crit Rev Toxicol 1997;27:299-318.
27. Sgard F, Charpantier E, Bertrand S, Walker N, Caput D, Graham D, et al. A novel human nicotinic receptor subunit, alpha10, that confers functionality to the alpha9-subunit. Mol Pharmacol 2002;61:150-9.
28. Lips KS, Konig P, Schatzle K, Pfeil U, Krasteva G, Spies M, et al. Coexpression and spatial association of nicotinic acetylcholine receptor subunits alpha7 and alpha10 in rat sympathetic neurons. J Mol Neurosci 2006;30:15-6.
29. Razani-Boroujerdi S, Boyd RT, Davila-Garcia MI, Nandi JS, Mishra NC, Singh SP, et al. T cells express alpha7-nicotinic acetylcholine receptor subunits that require a functional TCR and leukocyte-specific protein tyrosine kinase for nicotine-induced Ca2+ response. J Immunol 2007;179:2889-98.
30. Mishra NC, Rir-sima-ah J, Boyd RT, Singh SP, Gundavarapu S, Langley RJ, et al. Nicotine inhibits Fc epsilon RI-induced cysteinyl leukotrienes and cytokine production without affecting mast cell degranulation through alpha 7/alpha 9/alpha 10-nicotinic receptors. J Immunol 2010;185:588-96.
31. Dorion G, Israel-Assayag E, Beaulieu MJ, Cormier Y. Effect of 1,1-dimethylphenyl 1,4-piperazinium on mouse tracheal smooth muscle responsiveness. Am J Physiol Lung Cell Mol Physiol 2005;288:L1139-45.
32. Tournier JM, Birembaut P. Nicotinic acetylcholine receptors and predisposition to lung cancer. Curr Opin Oncol 2011;23:83-7.
33. Ellison M, Haberlandt C, Gomez-Casati ME, Watkins M, Elgoyhen AB, McIntosh JM, et al. Alpha-RgIA: a novel conotoxin that specifically and potently blocks the alpha9alpha10 nAChR. Biochemistry 2006;45:1511-7.
34. Whiteaker P, Christensen S, Yoshikami D, Dowell C, Watkins M, Gulyas J, et al. Discovery, synthesis, and structure activity of a highly selective alpha7 nicotinic acetylcholine receptor antagonist. Biochemistry 2007;46:6628-38.
35. Churg A, Cosio M, Wright JL. Mechanisms of cigarette smoke-induced COPD: insights from animal models. Am J Physiol Lung Cell Mol Physiol 2008;294: L612-31.
36. Murphy KM, Heimberger AB, Loh DY. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. Science 1990;250:1720-3.
37. Knutsen AP, Bush RK, Demain JG, Denning DW, Dixit A, Fairs A, et al. Fungi and allergic lower respiratory tract diseases. J Allergy Clin Immunol 2012;129: 280-91.
38. McGehee DS, Role LW. Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. Annu Rev Physiol 1995;57:521-46.
39. Lips KS, Luhrmann A, Tschernig T, Stoeger T, Alessandrini F, Grau V, et al. Down-regulation of the non-neuronal acetylcholine synthesis and release machinery in acute allergic airway inflammation of rat and mouse. Life Sci 2007;80: 2263-9.
40. Jones AK, Sattelle DB. Diversity of insect nicotinic acetylcholine receptor subunits. Adv Exp Med Biol 2010;683:25-43.
41. Sopori M. Effects of cigarette smoke on the immune system. Nat Rev Immunol 2002;2:372-7.
42. Jones AK, Sattelle DB. Functional genomics of the nicotinic acetylcholine receptor gene family of the nematode, *Caenorhabditis elegans*. Bioessays 2004;26:39-49.
43. Zhang J, Berg DK. Reversible inhibition of GABAA receptors by alpha7-containing nicotinic receptors on the vertebrate postsynaptic neurons. J Physiol 2007;579:753-63.
44. Jospin M, Qi YB, Stawicki TM, Boulin T, Schuske KR, Horvitz HR, et al. A neuronal acetylcholine receptor regulates the balance of muscle excitation and inhibition in *Caenorhabditis elegans*. PLoS Biol 2009;7:e1000265.
45. Rehan VK, Asotra K, Torday JS. The effects of smoking on the developing lung: insights from a biologic model for lung development, homeostasis, and repair. Lung 2009;187:281-9.
46. Carneiro J, Duarte L, Padovan E. Limiting dilution analysis of antigen-specific T cells. Methods Mol Biol 2009;514:95-105.
47. McCormick A, Loeffler J, Ebel F. *Aspergillus fumigatus*: contours of an opportunistic human pathogen. Cell Microbiol 2010;12:1535-43.
48. Verbitsky M, Rothlin CV, Katz E, Elgoyhen AB. Mixed nicotinic-muscarinic properties of the alpha9 nicotinic cholinergic receptor. Neuropharmacology 2000;39: 2515-24.
49. Profita M, Bonanno A, Montalbano AM, Ferraro M, Siena L, Bruno A, et al. Cigarette smoke extract activates human bronchial epithelial cells affecting non-neuronal cholinergic system signalling in vitro. Life Sci 2011;89:36-43.

METHODS

Cell cultures

NHBE cells were obtained from rejected lung transplant donor material and provided by Greg Connor of the University of Miami. Use of these cells was approved by the Institutional Review Board of Lovelace Respiratory Research Institute. Cells were grown at the ALI by using standard procedures.[E1] Briefly, cells were seeded into 100-mm collagen-coated plastic dishes (Corning, Inc, Corning, NY) and grown in bronchial epithelial cell growth medium (Lonza, Basel, Switzerland) supplemented with bovine pituitary extract, hydrocortisone (0.5 μg/mL), recombinant human epidermal growth factor (0.5 ng/mL), epinephrine (0.5 μg/mL), transferrin (10 μg/mL), insulin (5 μg/mL), retinoic acid (0.1 ng/mL), triiodothyronine (6.5 ng/mL), gentamicin sulfate (50 μg/mL), and amphotericin B (50 ng/mL). Cultures were maintained at 37°C in 5% CO_2, and the medium was changed every alternate day until the cells reached approximately 90% confluence. Cells were harvested, and 2.5×10^5 cells were seeded onto a 12-mm collagen-coated Corning Costar Transwell-Clear insert with 0.4-μm pores and submerged in the differentiation medium (1:1 Dulbecco modified Eagle medium and bronchial epithelial cell growth medium with the above supplements except gentamicin sulfate and amphotericin B and with 50 nmol/L all-trans retinoic acid and 1.5 μg/mL BSA added). After 7 days, the medium was removed from the top side of the insert to allow cell differentiation and establish the ALI. The culture medium was replaced every alternate day, and the apical surfaces of the cells were rinsed with PBS once every week. Alternatively, predifferentiated NHBE cells (EpiAirway Tissue Model) were purchased from Mattek (Ashland, Mass). A549 cells (CCL-185) were purchased from ATCC (Manassas, Va) and grown according to the vendor's directions.

Animal studies

OVA-TCR transgenic hemizygous mice (DO11.10) on a BALB/c background were bred in our animal facility by mating DO11.10 +/− hemizygous males with BALB/c females. Mice were exposed in whole-body inhalation exposure chambers (Hazelton H2000, Lab Products, Maywood, NJ) to air, SS, or nicotine (1.5 mg total particulate material/m^3), heat-aggregated OVA aerosol (5 mg/m3), or OVA plus SS or nicotine for 2 weeks (6 h/d for 5 d/wk). In some experiments normal (wild-type) BALB/c mice were sensitized to *A fumigatus* (Greer Laboratories, Lenoir, NC) extract, as described previously.[E2] Briefly, the allergen used in the study was a lyophilized culture filtrate preparation of *A fumigatus*; the filtrates were stored at −70°C until use. Mice were immunized intratracheally with *A fumigatus* extract (50 mg/0.1 mL of endotoxin-free sterile saline) or sterile saline alone and subsequently challenged with the *A fumigatus* extract (100 mg/0.1 mL administered intratracheally) 3 times at 5-day intervals. For MM treatment, mice were subcutaneously implanted with MM-containing miniosmotic pumps 3 weeks before exposures, as previously described.[E3] The pumps delivered 2 mg of MM/kg body weight per day. The trachea was surgically exposed and cannulated and the right lobe was lavaged twice with 1 mL of sterile Ca^{2+}/Mg^{2+}-free PBS (pH 7.4) to collect lung lavage fluid. Total and differential BAL cells were counted microscopically.[E2] The left lung was fixed and the right lung was processed for RNA and protein extraction.[E4] All animal protocols used in this study were approved by the Institutional Animal Care and Use Committee of the Lovelace Respiratory Research Institute.

Mucus staining and IHC

NHBE cells at the ALI were fixed in 10% neutral buffered formalin and embedded in paraffin; 5-μm-thick sections were stained for mucus with AB/PAS.[E5,E6] Briefly, after deparaffinization, the slides were stained with AB solution at pH 2.5 for 30 minutes, and after washing, they were treated with 1% periodic acid for 10 minutes. The slides were stained with Schiff reagent for 10 minutes, rinsed 3 times with sodium metabisulfite, washed, dehydrated, mounted, and examined microscopically at the indicated magnification. To quantify mucus-producing cells and mucus volume, we determined the number of AB/PAS-positive cells per millimeter of basal lamina and the mucus volume per cubic meter of basement membrane. The tissue sections were examined and quantified blind by using an Olympus BH-2 light microscope (Olympus, Center Valley, Pa) equipped with the National Institutes of Health image analysis system, as described previously.[E6]

Immunohistochemical staining was performed according to the protocol previously described.[E7] Mouse monoclonal anti-MUC5AC (45M1; Thermo Scientific, Lafayette, Colo) and rabbit polyclonal anti-$GABA_AR\alpha2$ (Sigma, St Louis, Mo) antibodies were used at preoptimized concentrations of 1:100 and 1:50 dilution, respectively. The tissue sections were treated with the primary antibodies overnight at 4°C, followed by incubation with biotinylated secondary antibody (VECTASTAIN Elite ABC kit; Vector Laboratories, Burlingame, Calif). Binding was visualized with an avidin-biotinylated enzyme complex (VECTASTAIN Elite ABC kit), with 3, 3′-diaminobenzidine as substrate.

Cell treatments

Cells were treated with 100 nmol/L nicotine base (Sigma), 10 to 50 ng/mL recombinant human IL-13 (R&D Systems, Minneapolis, Minn), or indicated concentrations of NB, respectively, and the cultures were harvested 48 hours later. $GABA_AR$, nAChR, or muscarinic receptor inhibitors were added at the indicated concentrations 2 hours before the addition of IL-13, nicotine, or NB.

RT-PCR and qPCR

Total RNA from murine lungs, NHBE cells, and A549 cells was isolated by using TRI-Reagent. One-step RT-PCR was performed with the SuperScript III One-Step RT-PCR System with Platinum Taq. RT-PCR primers for $GABA_AR\alpha2$ were 5′-AGGCTTCCGTTATGATACAG (forward) and 5′-AGGACTGACCCCTAATACAG (reverse), and those for GAPDH were 5′-CCCATCACCATCTTCCAGGAG (forward) and 5′-TTCACCACCTTCTTCTTGATGTCAT (reverse); primers were purchased from Sigma.

qPCR was performed with the Step One plus Detection System (Applied Biosystems, Foster City, Calif) and the TaqMan One-Step RT-PCR kit containing AmpliTaq Gold DNA polymerase. Specific primers and probes for MUC5AC; the nAChR α7/α9/α10 subunits; muscarinic receptors M1, M2, M3; and $GABA_AR\alpha2$ (forward 5′-CCACCATCTCCAAGAGTGCAA and reverse 5′-TGCTTCAGCTGGCTTGTTTTC and probe CACGCCAGAACCCAACAAGAAGCC) for qPCR were obtained from Applied Biosystems. Fold differences were determined by using the $2^{(-\Delta\Delta CT)}$ method.[E8]

Knockdown of α7-, α9-, and α10-nAChRs with siRNAs

siRNAs were purchased from Thermo Scientific. The siRNA SMARTpool contained 4 pooled siRNA duplexes with "UU" overhangs and a 5′ phosphate on the antisense strand. Briefly, the siRNA-DharmaFECT 1 complex was added to the cells in complete medium and incubated at 37°C in 5% CO_2 for 48 hours. The mRNA levels of α7-, α9-, and α10-nAChRs were assessed 48 hours after transfection with TaqMan One-Step RT-PCR. Protein levels of $GABA_AR\alpha2$ were determined by using Western blot analysis.

Western blot analysis

Western blot analysis was performed as described previously.[E7] Briefly, lung tissues were homogenized in RIPA buffer (20 mmol/L Tris, 150 mmol/L NaCl, 20 mmol/L β-glycerol-phosphate, 1% Triton-X, 10 mmol/L NaF, 5 mmol/L EDTA, and 1 mmol/L Na_3VO_4) containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride and 1 μg/mL each of aprotinin, antipain, and leupeptin) at 4°C. The protein content of the extracts was determined by using the BCA Protein Assay Kit (Pierce, Rockford, Ill). Samples were electrophoresed on 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, Calif). Nonspecific binding was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) for 30 minutes at room temperature, followed by overnight incubation with the indicated specific primary antibody at 4°C. $GABA_AR\alpha2$ expression was determined by probing the blot with anti-$GABA_AR$ α2 murine mAb (Millipore, Temecula, Calif). The blots were washed with TBST (3×), incubated for 1 hour at room temperature with horseradish peroxidase–conjugated secondary antibody, and washed with TBST (3×). Blots were developed

with ECL (GE Healthcare, Hertfordshire, United Kingdom) with x-ray photo film.

Statistical analysis

All data were analyzed with GraphPad Prism software 5.03. One-way ANOVA was used to compare means between the groups by using the Tukey *post hoc* test, which compares all groups at 95% CIs. Results are presented as means ± SDs. Differences with a P value of less than .05 were considered statistically significant.

REFERENCES

E1. Atherton HC, Jones G, Danahay H. IL-13-induced changes in the goblet cell density of human bronchial epithelial cell cultures: MAP kinase and phosphatidylinositol 3-kinase regulation. Am J Physiol Lung Cell Mol Physiol 2003;285: L730-9.

E2. Singh SP, Gundavarapu S, Pena-Philippides JC, Rir-Sima-Ah J, Mishra NC, Wilder JA, et al. Prenatal secondhand cigarette smoke promotes th2 polarization and impairs goblet cell differentiation and airway mucus formation. J Immunol 2011; 187:4542-52.

E3. Singh SP, Kalra R, Puttfarcken P, Kozak A, Tesfaigzi J, Sopori ML. Acute and chronic nicotine exposures modulate the immune system through different pathways. Toxicol Appl Pharmacol 2000;164:65-72.

E4. Singh SP, Mishra NC, Rir-Sima-Ah J, Campen M, Kurup V, Razani-Boroujerdi S, et al. Maternal exposure to secondhand cigarette smoke primes the lung for induction of phosphodiesterase-4D5 isozyme and exacerbated Th2 responses: rolipram attenuates the airway hyperreactivity and muscarinic receptor expression but not lung inflammation and atopy. J Immunol 2009;183: 2115-21.

E5. El-Zimaity HM, Ota H, Scott S, Killen DE, Graham DY. A new triple stain for Helicobacter pylori suitable for the autostainer: carbol fuchsin/Alcian blue/hematoxylin-eosin. Arch Pathol Lab Med 1998;122:732-6.

E6. Harkema JR, Hotchkiss JA. In vivo effects of endotoxin on intraepithelial mucosubstances in rat pulmonary airways. Quantitative histochemistry. Am J Pathol 1992;141:307-17.

E7. Mishra NC, Rir-Sima-Ah J, Langley RJ, Singh SP, Pena-Philippides JC, Koga T, et al. Nicotine primarily suppresses lung Th2 but not goblet cell and muscle cell responses to allergens. J Immunol 2008;180:7655-63.

E8. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25: 402-8.

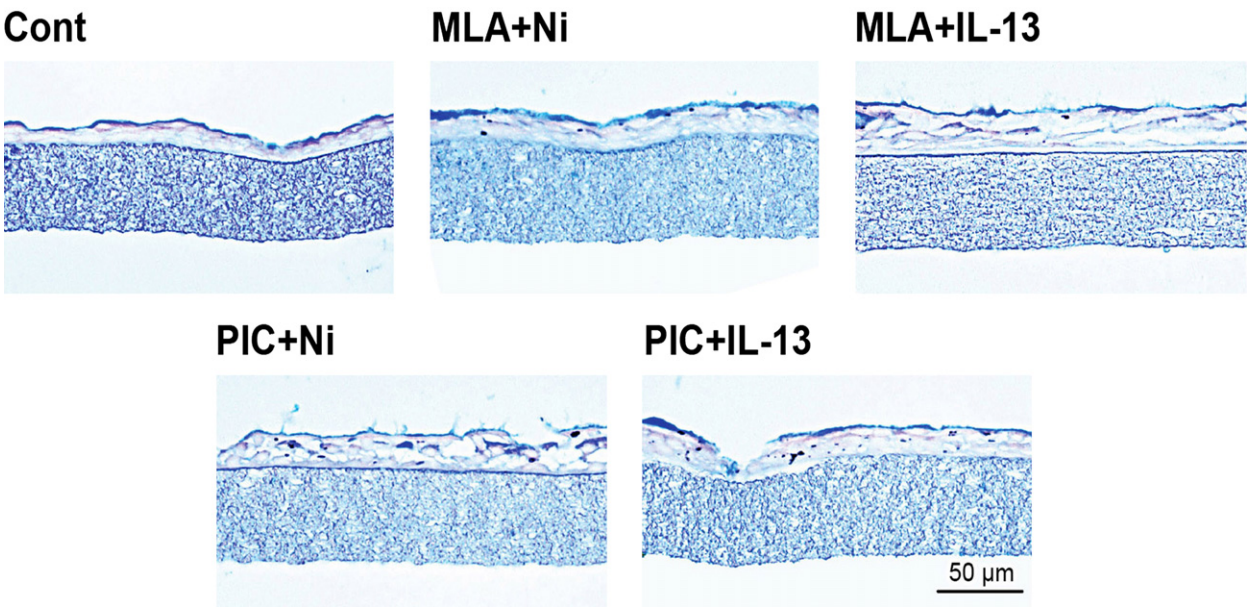


FIG E1. MLA and PIC block the nicotine *(Ni)*– and IL-13–induced mucus formation in NHBE cells. NHBE cells were cultured at the ALI with 100 nmol/L nicotine and 50 ng/mL IL-13 for 48 hours. Where indicated, 1 μmol/L MLA or 50 μmol/L PIC was added to cultures 2 hours before nicotine, IL-13, or both. The *inserts* were fixed and stained for mucus (AB/PAS).

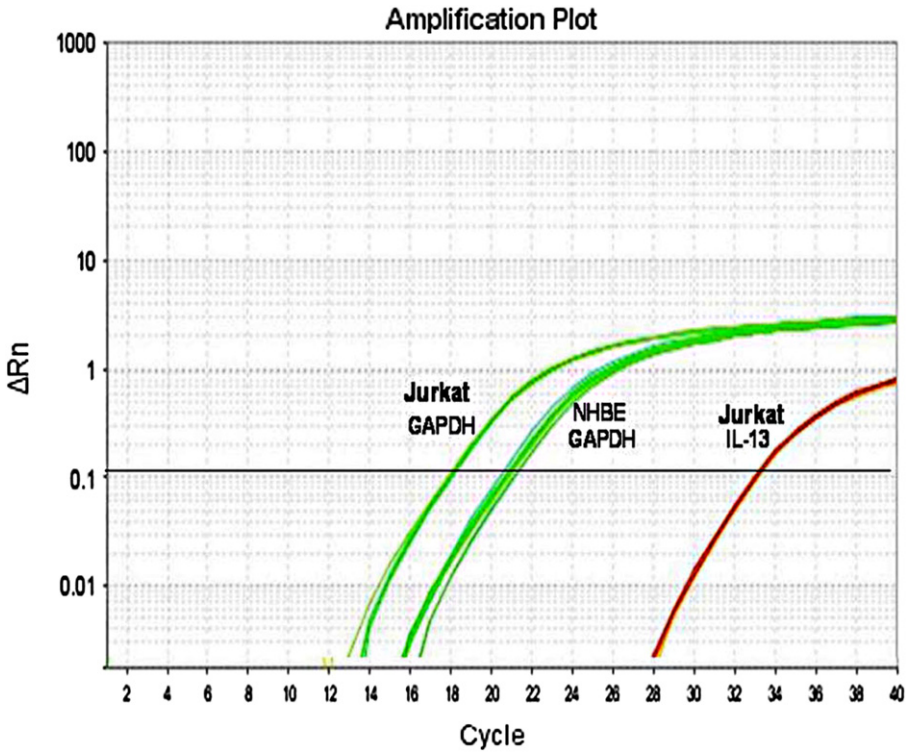


FIG E2. Unlike human Jurkat T cells, NHBE cells do not express the IL-13 gene, even after nicotine treatment. RNA was isolated from Jurkat cells and NHBE cells (with and without 100 nmol/L nicotine treatment for 48 hours) and analyzed for the expression of IL-13 by using qPCR. The experiments were repeated 3 times with different batches of RNA. *ΔRn*, The magnitude of the signal generated by the given set of PCR conditions.

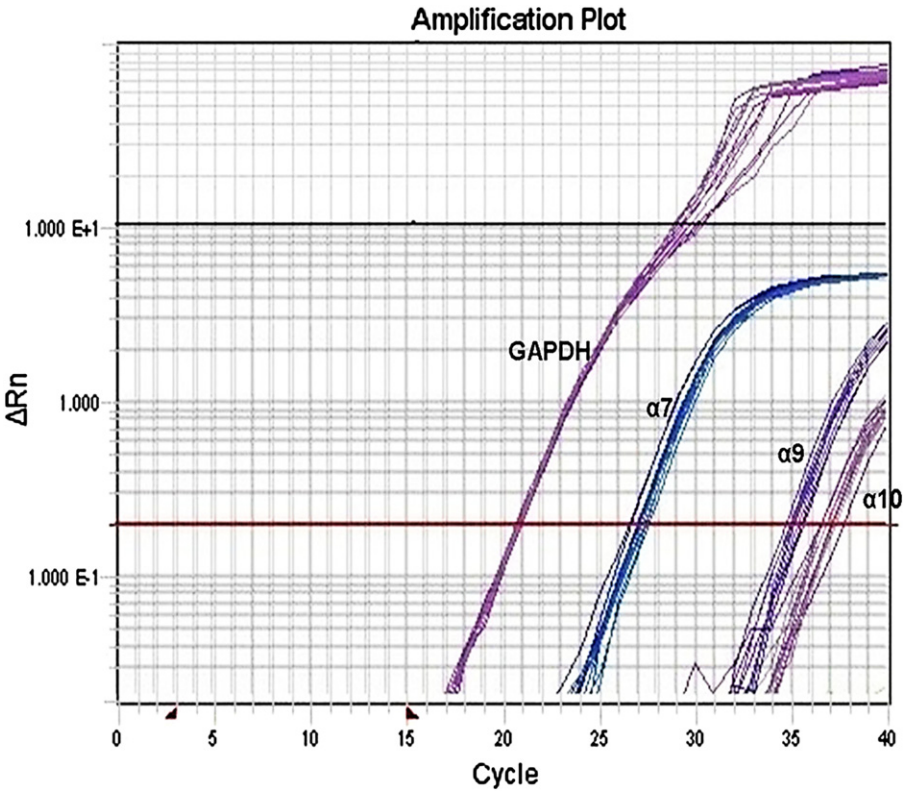


FIG E3. NHBE cells express α7-, α9-, and α10-nAChR subunits. RNA from NHBE cells was analyzed for the expression of α3-, α4-, α5-, α7-, α9-, α10-, and β2-nAChR subunits by using qPCR. Only the α7, α9, and α10 subunits were detected within 40 cycles of amplification. *ΔRn*, The magnitude of the signal generated by the given set of PCR conditions.

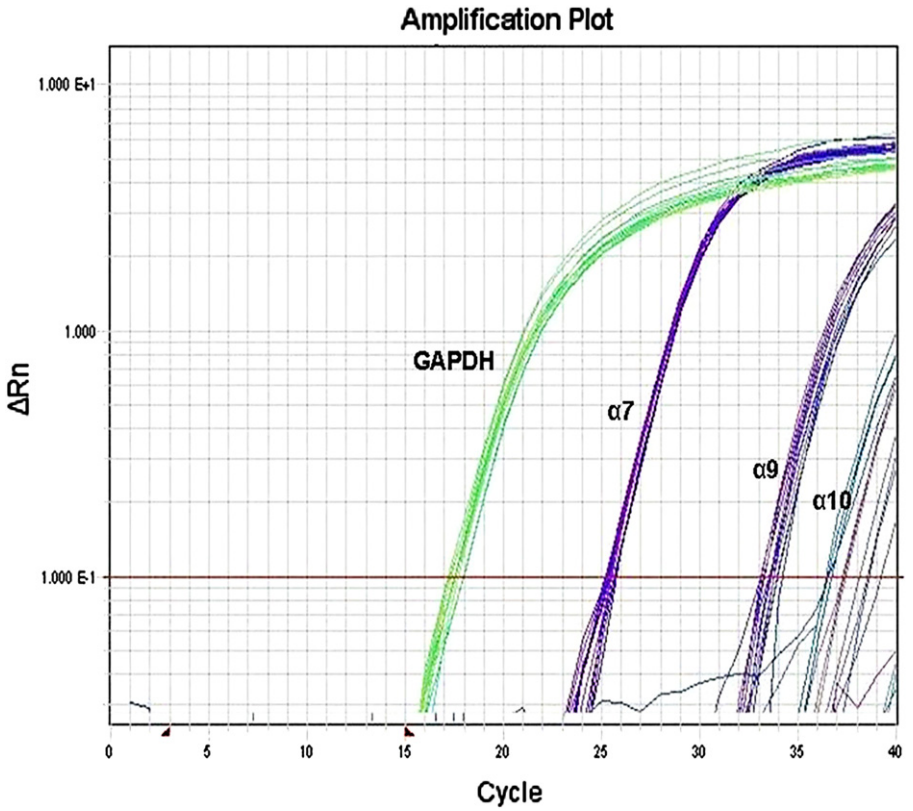


FIG E4. A549 cells express α7-, α9-, and α10-nAChR subunits. RNA from A549 contained mRNA for α7-, α9-, and α10-nAChR subunits, as detected by using qPCR. However, these cells also contained detectable levels of α3, α4, and β2 subunit mRNA (not shown). *ΔRn*, The magnitude of the signal generated by the given set of PCR conditions.

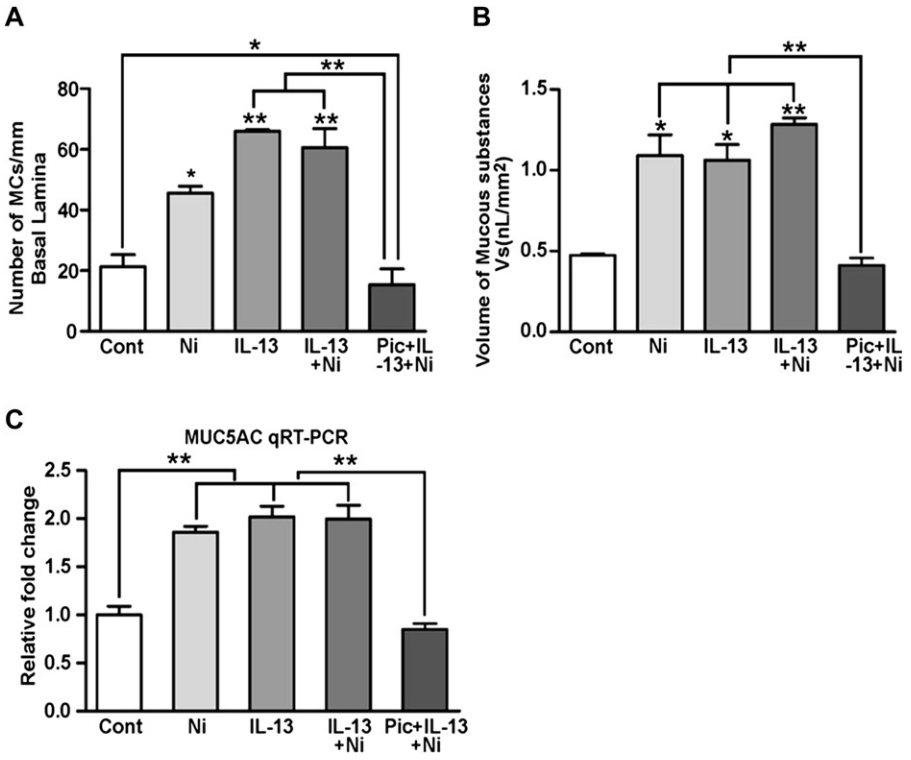


FIG E5. Picrotoxin blocks the effects of nicotine *(Ni)* and IL-13 on mucus formation. NHBE cells were treated with nicotine, IL-13, or both, as described in the Methods section. In some cultures 50 μmol/L PIC was added 2 hours before IL-13 and nicotine. The number of mucus-containing cells per millimeter of basal lamina **(A)**, the volume of mucus-positive cells **(B)**, and MUC5AC expression determined by means of qPCR **(C)** in the presence and absence of PIC are shown. *Bars* represent values ± SEMs for 3 inserts. Each experiment was repeated at least 5 times. $*P \leq .05$ and $**P \leq .01$.

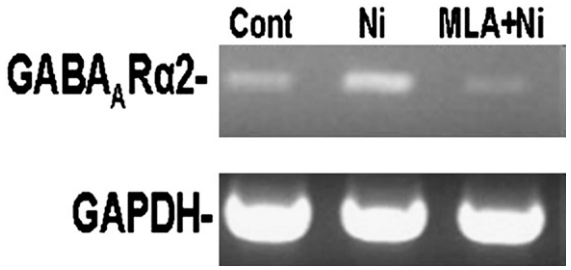


FIG E6. MLA blocks nicotine *(Ni)*–induced $GABA_AR2\alpha$ in A549 cells. mRNA from A549 cells after treatment with nicotine and nicotine plus MLA (1 μmol/L) was analyzed by using RT-PCR, as described in the Methods section.

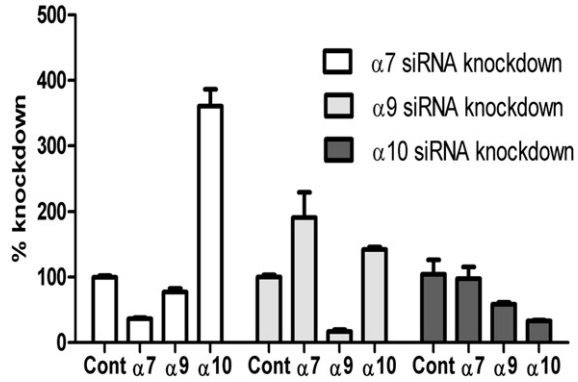


FIG E7. Specific siRNAs decrease the mRNA expression of the selected nAChR subtypes. A549 cells were treated with α7-, α9-, and α10-specific siRNA, as described in the Methods section. At 48 hours after siRNA treatment, the expression of the α7-, α9-, and α10-nAChR subunits was assayed by using qPCR. The increased expression of the α10 subunit after α7 siRNA treatment was consistently observed. The experiment was repeated twice. *Bars* represent means ± SEMs from 2 separate siRNA-treated cell cultures.

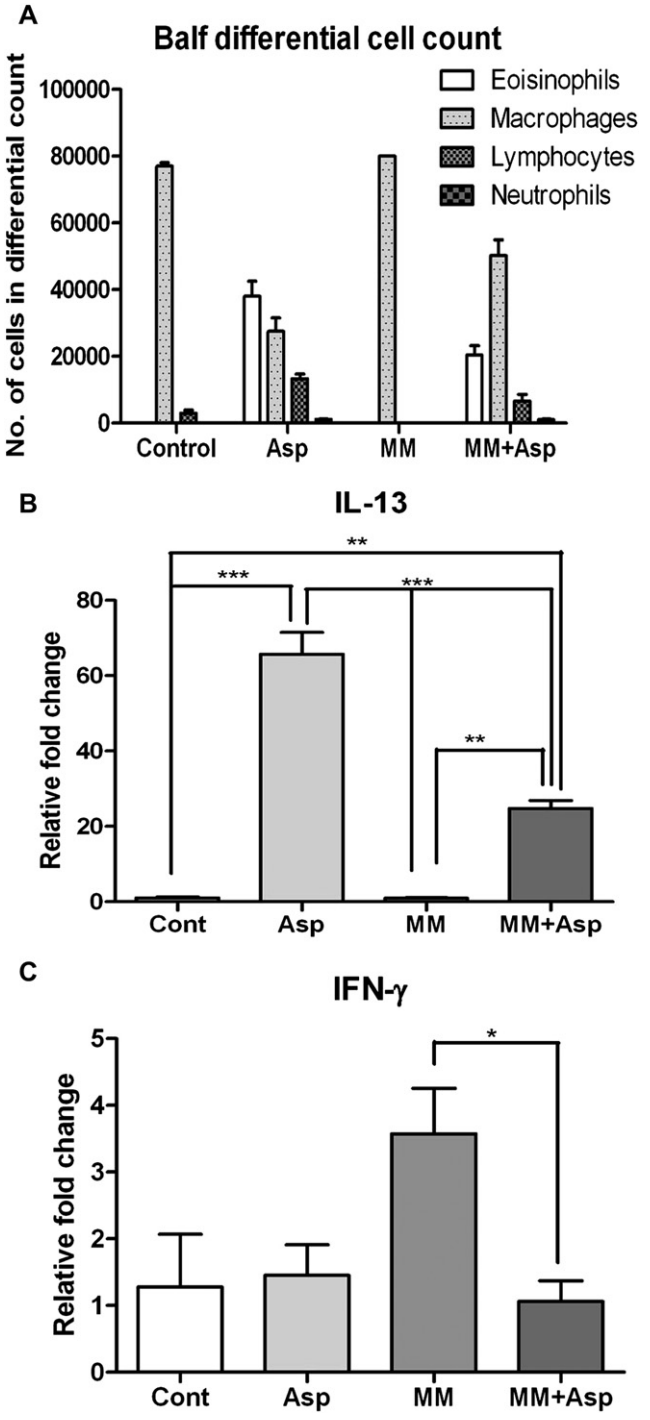


FIG E8. *Aspergillus* species *(Asp)* induce MM-sensitive $GABA_AR\alpha 2$ and mucus formation in the airways. BALB/c wild-type mice were exposed to *Aspergillus fumigatus* extract, as described in the Methods section. Some animals received the nAChR inhibitor MM. Differential cell counts in bronchoalveolar lavage fluid **(A)**, lung IL-13 expression determined by using qPCR **(B)**, and lung IFN-γ expression determined by using qPCR in various groups **(C)** are shown. The results represent 2 independent *in vivo* exposure experiments with 3 to 6 mice per group. $*P \leq .05$, $**P \leq .01$, and $***P \leq .001$.

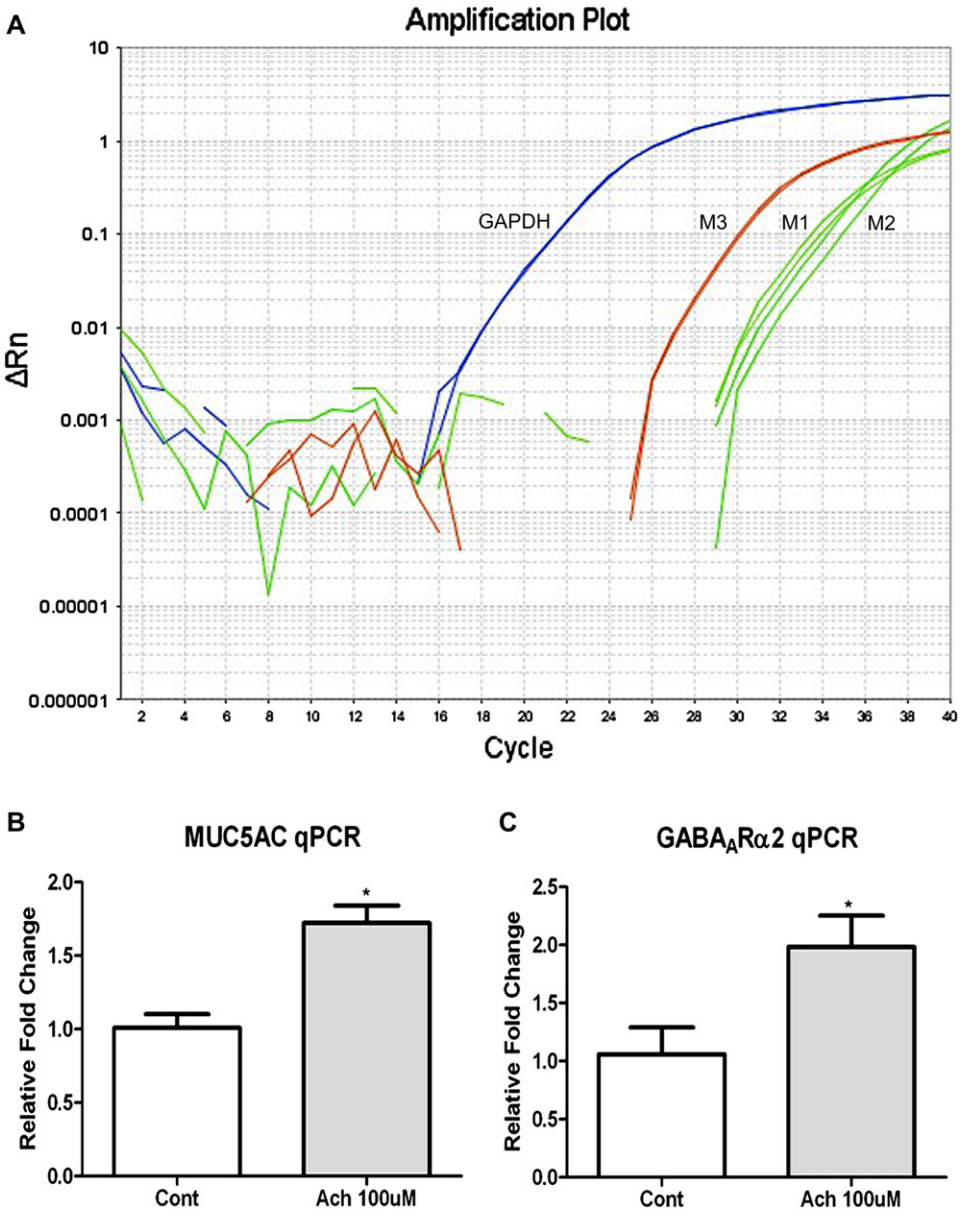


FIG E9. NHBE cells express M1, M2 and M3 muscarinic receptor subunits. **A,** RNA from NHBE cells was analyzed for the expression of M1, M2, and M3 muscarinic subunits by using qPCR. **B,** Acetylcholine (*Ach*; 100 μmol/L) was added to NHBE cells, and 48 hours later, the cells were analyzed for MUC5AC and $GABA_AR\alpha2$ mRNA expression by using qPCR. *ΔRn*, The magnitude of the signal generated by the given set of PCR conditions. $*P \leq .05$.

Addendum to the previous report

Introduction: The previous report was rejected because we did not explain why we made changes in the specific aim 1 and how the report addressed the specific aims in the application. This is an addendum to the previously submitted report that provides the rationale for making adjustments to aim 1 and the summary of the results obtained.

Reasons for deviating from specific aim 1

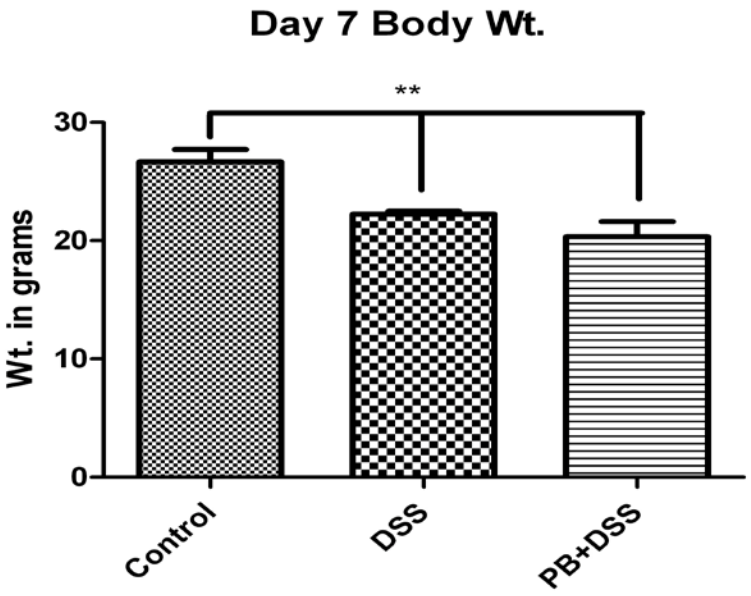
The application had 3 specific aims to determine: (1) role of nicotinic acetylcholine receptors (nAChRs) and muscarinic receptors in sarin-induced immunosuppression, (b) effects of sarin/pyridostigmine bromide (PB) in infection/autoimmune disease, and (c) efficacy of potential therapeutic interventions. Aim 1 proposed to use subclinical levels of sarin exposure to raise acetylcholine levels and determine its effects on immunological responses. In our previous studies, we used to obtain sarin from the US Army; however, because of security issues lending to very high charges for delivery ($30,000/shipment), we opted to synthesize sarin in house. The Lovelace Respiratory Research Institute (LRRI) decided to seek approval to make small batches of sarin and, by various analytic tests, succeeded to synthesize sarin. Unfortunately, when we exposed the animals to putative subclinical doses of sarin, animals got sick and died within 36 hr of inhalation. In the second trail, with reduced dosage, more than half of the animals died. Death in these animals was due to severe bronchoconstriction and lung epithelial cell damage (Gundavarapu et al., unpublished observation). Thus the lung is a major target of sarin, and because epithelial cells in the lung play an important role immune/inflammatory responses (*1*), effects of sarin on these cells could contribute to the immunological effects of sarin. Because it costs enormous amount of money to expose animals to compounds like sarin together with uncertainty about sarin exposure dose, we decided to use cell culture models to ascertain the effects of cholinergic compounds and cholinergic receptors on immune/inflammatory responses. We opted to use normal human bronchial epithelial (NHBE) cells and cell lines to study the effects of cholinergic agents and cholinergic receptors on mucus responses. Indeed, NHBE cells have nicotinic (*2*), muscarinic (*3*), and gamma aminobutyric acid $(GABA)_A$-receptors (*4, 5*), specifically $GABA_AR2\alpha$ (*2*). Importantly, we found that acetylcholine or acetylcholinesterase inhibitors – neostigmine and pyridostigmine bromide induce mucus formation in NHBE cells (*2*). This may explain how cholinergic compounds including sarin activate the airway secretory cells and affect the pulmonary immune function. These experiments are detailed in the original report and appendices.

Mucus is an excellent milieu for bacterial growth (*6*) and experiments are planned to test whether chronic exposure to pyridostigmine bromide (PB) exacerbates Legionella infections.

Specific Aim 2

To evaluate the effects of cholinergic compounds on chronic inflammatory bowel diseases, we used the mouse model of ulcerative colitis (7 day exposure to dextran sulfate). The experimental groups included: control, dextran sulfate, and PB + dextran sulfate. Dextran sulfate was given to the animals through drinking water and PB by subcutaneously implanted Alzet miniosmotic pumps. The experiment has been completed and we are awaiting the pathology reports and immunologic analysis to determine whether PB exacerbates the disease. Animals were also weighed before and after dextran sulfate and PB treatment. Animal weights showed that dextran sulfate and dextran sulfate + PB-treated groups had significantly lower body weights than control animals. Body weights of the animals in PB + dextran sulfate groups over dextran sulfate alone tended to be lower but did not reach statistical significance (see Addendum Fig. 1). Hopefully, in the next few weeks we will have immunological and pathological data to determine whether acetylcholine esterase inhibitors exacerbate both

respiratory and gastrointestinal diseases – two of the symptoms associated with the Gulf War syndrome.



Addendum Fig.1: *Three groups ofC57Bl/6 mice (8 animals/group) representing control, dextran sulfate (DDS), and dextran sulfate + PB were started for this experiment. Dextran sulfate (3% in drinking water) was provided by drinking water for 7 days. One week prior to dextran sulfate, the dextran sulfate + PB group were implanted subcutaneously with PB (2mg/kg/body wt.concentration?)-containing miniosmotic pumps. The experiment was stopped after 1-week treatment with dextran sulfate and body weights recorded before sacrifice.*

Specific aim 3

Specific aim 3 was directed to test potential interventions. We already showed that airway mucus formation in response to cholinergic agents such as neostigmine and nicotine are blocked by nicotinic receptor antagonists in cell culture. *In vivo* mucus formation in response to nicotine or cigarette smoke is also inhibited by nicotinic acetylcholine receptor inhibitors (*2*). Recently, we have identified HIV gp120 as a strong pro-mucoid agent in the airways (*7*); (see the new appendix – to be submitted to Journal of Experimental Medicine). This may explain the epidemiological data indicating a significantly increased incidence of obstructive pulmonary diseases in HIV-infected US veterans (*8*). After establishing the gut pathology associated with dextran sulfate + PB, we will determine whether anticholinergic agents ameliorate the dextran sulfate and/or dextran sulfate + PB gut pathology.

References in the addendum

1. **J. D. Crapo, A. G. Harmsen, M. P. Sherman, R. A. Musson, Pulmonary immunobiology and inflammation in pulmonary diseases. *American journal of respiratory and critical care medicine* 162, 1983 (Nov, 2000).**

2. **S. Gundavarapu *et al.*, Role of nicotinic receptors and acetylcholine in mucous cell metaplasia, hyperplasia, and airway mucus formation in vitro and in vivo. *The Journal of allergy and clinical immunology* 130, 770 (Sep, 2012).**
3. **M. Profita *et al.*, Cigarette smoke extract activates human bronchial epithelial cells affecting non-neuronal cholinergic system signalling in vitro. *Life Sci* 89, 36 (Jul 4, 2011).**
4. **X. W. Fu, K. Wood, E. R. Spindel, Prenatal nicotine exposure increases GABA signaling and mucin expression in airway epithelium. *American journal of respiratory cell and molecular biology* 44, 222 (Feb, 2011).**
5. **Y. Y. Xiang *et al.*, A GABAergic system in airway epithelium is essential for mucus overproduction in asthma. *Nature medicine* 13, 862 (Jul, 2007).**
6. **J. A. Voynow, B. K. Rubin, Mucins, mucus, and sputum. *Chest* 135, 505 (Feb, 2009).**
7. **S. Gundavarapu *et al.*, Role of gp120 in HIV-induced airway mucus formation. *Manuscript Submitted*, (2013).**
8. **K. Crothers *et al.*, Increased COPD among HIV-positive compared to HIV-negative veterans. *Chest* 130, 1326 (Nov, 2006).**

Appendix

Title: Role of gp120 in HIV-induced airway mucus formation

Sravanthi Gundavarapu[1], Neerad C. Mishra[1], Shashi P. Singh[1], Raymond Langley[1], Ali Imran Saeed[2], Carol A. Feghali-Bostwick[3], J. Michael McIntosh[4], Julie Hutt[1], Shilpa Buch[5], and Mohan L. Sopori[1]

[1]Respiratory Immunology Division, Lovelace Respiratory Research Institute, Albuquerque, NM; [2]Pulmonary and Critical Care Medicine, University of New Mexico, Albuquerque, NM; [3]Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA; [4]Departments[4]George E. Wahlen Veterans Affairs Medical Center, Salt Lake City, UT and Departments of Psychiatry and Biology, University of Utah, Salt Lake City, UT; [5]Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE;

Corresponding Author

Mohan Sopori, Ph.D.

Respiratory Immunology Division

Lovelace Respiratory Research Institute,

Albuquerque, NM 87108

Email: msopori@lrri.org

Tel: (505) 348-9440

Fax: (505) 348-4986

Short Title: Gp120 and airway mucus formation

Abstract

Lung diseases such as asthma, chronic bronchitis, chronic obstructive pulmonary disease, and lung infections are a major cause of morbidity and mortality among HIV-infected patients even in the era of antiretroviral therapy (ART). Excessive airway mucus formation is a characteristic of these diseases and contributes to airway obstruction and lung infections. HIV-gp120 is an important factor in HIV pathogenesis and normal human bronchial epithelial (NHBE) cells which make airway mucus, express the HIV-gp120 coreceptor CXCR4 but not CCR5, and CXCR4-tropic and CXCR4/CCR5 dual-tropic HIV-gp120 induced mucus production in NHBE cells, which was blocked by CXCR4 but not CCR5 inhibitors. Airways mucus is regulated by α7-nicotinic acetylcholine receptor (α7-nAChR) and epithelial growth factor receptor (EGFR) pathways. The gp120-induced mucus formation was inhibited by α7-nAChR but not EGFR inhibitors. To ascertain the *in vivo* relevance of these results, we examined HIV-infected and simian immunodeficiency virus (SIV)-infected postmortem lung tissues for mucus and gp120 expression. HIV- and SIV-infections, even after ART, led to gp120 immunoreactivity and mucus formation in the lung. These results suggest, for the first time, that HIV-gp120 promotes mucus formation that may contribute to pulmonary obstructive diseases in HIV-infected subjects. (**Word count 189**)

Introduction

Prior to the advent of anti-retroviral therapy (ART), pulmonary diseases were among the most frequent complications of HIV infection (Murray and Mills, 1990); however, while HIV-associated mortality has decreased substantially after the introduction of anti-retroviral therapy (ART) (Palella et al., 1998), lung diseases continue to remain a major cause of morbidity and mortality among HIV patients (Grubb et al., 2006). HIV-infected patients exhibit a significantly higher incidence and early onset of chronic obstructive pulmonary disease (COPD), chronic bronchitis, asthma and lung infections (Diaz et al., 2000; Gingo et al., 2010; Morris et al., 2011). For example, it was reported that 23% of relatively young (mean age 34 years) HIV-infected smokers without a history of pulmonary infections developed COPD/emphysema as detected by computer tomography scan and lung function testing, compared to only 2% of control subjects matched for age and smoking history, and the participants were relatively healthy (Diaz et al., 2000). The most common respiratory symptoms in these patients was cough, airway mucus, dyspnea and respiratory infections (Diaz et al., 2003). The mechanism(s) by which HIV infection promotes lung disease in the presence of ART is not clear; however, even under conditions of controlled HIV viremia, the virus may persist in reservoirs leading to low levels of viral RNA and/or proteins (Rychert et al., 2010). The HIV envelop glycoprotein gp120 may be present in the plasma, lymphoid tissue, and brain of HIV-infected patients and simian immunodeficiency virus (SIV)-infected monkeys before and after ART (Rychert et al., 2010; Santosuosso et al., 2009). Moreover, lungs may harbor significant levels of the latent virus (North et al., 2010) and pulmonary infections may activate the latent virus (Segal et al., 2011).

Airway mucus overproduction is a common characteristic of lung diseases such as chronic bronchitis, COPD, and asthma. While airway mucus plays an important role in mucociliary clearance and is the first line of defense against inhaled pathogens and particulate matter (Hovenberg et al., 1996), excessive mucus formation contributes to airway obstruction and pathogenesis of COPD, airway inflammation, asthma, and chronic bronchitis (Rose and Voynow, 2006). Excessive mucus is also an excellent milieu for bacterial growth and encourages lung infections (Voynow and Rubin, 2009). We and others have demonstrated that airway mucus formation is strongly influenced by gamma aminobutyric acid $(GABA)_AR\alpha2$ and nicotinic acetylcholine receptors (nAChRs) in the airway epithelial cells (Fu et al., 2011; Gundavarapu et al., 2012; Xiang et al., 2007), and reciprocally, nAChR antagonists suppress allergen and cigarette smoke (CS)/nicotine-induced airway mucus formation both *in vitro* and *in vivo* (Gundavarapu et al., 2012). In this communication we present evidence that HIV gp120 induces mucus formation in normal human bronchial epithelial (NHBE) cells through the HIV co-receptor CXCR4 using the α7-nAChR - $GABA_AR\alpha2$ pathway but not the epithelial growth factor receptor (EGFR) pathway. Moreover, even after ART, autopsied lungs tissues from HIV and simian immunodeficiency virus (SIV) infected humans and monkeys, respectively, show the presence of gp120, mucus, and $GABA_AR\alpha2$ expression.

Results and Discussion

HIV gp120 promotes mucus formation in NHBE cells: Although a large percentage of HIV-infected patients are smokers (Forey et al., 2011; Niaura et al., 2000) and CS is the major risk factor in many lung diseases (Forey et al., 2011), HIV may be an independent risk factor for COPD, asthma, and bronchitis (Gingo et al., 2010; Raynaud et al., 2011), and compared to HIV-negative veterans, HIV-positive veterans with similar smoking history are 50-60% more likely to develop COPD (Crothers et al., 2006). To understand the manner by which HIV promotes airway mucus formation, we ascertained whether HIV gp120 plays a role in airway mucus formation. The rationale for focusing on gp120 were: (a) the bronchoalveolar lavage cells from HIV patients with lung infections secrete gp120 (Rubbo et al., 2011), (b) significant levels of gp120 are found in plasma and tissues of HIV-infected patients even after highly active ART (HAART) (Rychert et al., 2010; Santosuosso et al., 2009), and (c) gp120 has been implicated in number of HIV-related pathologies including neuropathogenesis and vascular abnormalities (Kanmogne et al., 2005; Kaul et al., 2001). In the airways, mucus is formed by goblet cells – a subgroup of airway epithelial cells (Hovenberg et al., 1996), and we determined whether gp120 in realistic concentrations triggered mucus formation in NHBE cells grown on air-liquid interphase (Gundavarapu et al., 2012). We used a concentration of 10-100 ng/ml of X4-gp120 ($gp120_{LAV}$) in NHBE cell cultures. The rationale for using these concentrations was that many HIV patients on HAART have gp120 levels in plasma/tissues that are within this concentration range and some may even reach 796 ng/ml (Rychert et al., 2010). Fig. 1A shows that as little as 10 ng/ml of gp120 induces detectable mucus in NHBE cells. Mucus content was optimal around 100

ng/ml of gp120 and, unless mentioned otherwise, we used $gp120_{LAV}$ (gp120) at 100 ng/ml in NHBE cell cultures.

HIV gp120 induces mucus through CXCR4 receptors on NHBE cells: CD4 is the major receptor for HIV entry (Wilen et al., 2012); however, NHBE cells do not express CD4 (not shown); therefore, we determined whether gp120 affected mucus formation through CXCR4 (X4) or CCR5 (R5) HIV coreceptors. We used gp120 variants, including $gp120_{LAV}$ (X4-tropic), $gp120_{MN}$ (R5X4 dual-tropic), and $gp120_{ZM}$ (R5-tropic). Compared to the R5-tropic $gp120_{ZM}$, X4-tropic $gp120_{LAV}$ and R5X4-tropic $gp120_{MN}$ strongly induced mucus formation and mucous cell metaplasia in NHBE cells (Fig. 1B). Similarly, as detected by qPCR, the expression of MUC5AC (Fig. 1C, left panel) - the major inducible mucin in the airway mucus and $GABA_AR\alpha2$ (Fig. 1C, right panel) was upregulated more strongly by X4- and X4/R5- than R5-specific gp120, suggesting gp120-X4 is a strong stimulator of mucus formation in NHBEC. Because even the R5-tropic $gp120_{ZM}$ modestly increased mucus formation in NHBE cells, we determined whether NHBE cells expressed CXCR4 and/or CCR5. CXCR4 plays a critical role in allergic inflammation (Gonzalo et al., 2000) and its expression was previously reported on bronchial epithelial cells (Eddleston et al., 2002); persistent high expression of CXCR4 was also noted in the lungs of HIV patients on HAART (Tavares Marques, et al., 2007). We used qPCR to ascertain the presence of CXCR4 and CCR5 on NHBE cells. Results presented in Fig. 2A indicate that NHBE cells express CXCR4 but the expression of CCR5 was not detectable up to 40 cycles of qPCR analysis. Thus, the small amount of mucus induced by $gp120_{ZM}$ in NHBE cells may reflect some degree of crossreactivity of $gp120_{ZM}$ with

CXCR4. The induction of mucus by $gp120_{LAV}$ (Fig. 2B), and $gp120_{MN}$ and $gp120_{ZM}$ (not shown) was blocked by pretreatment of NHBE cells with CXCR4 antagonist AMD3100, but not by the CCR5 antagonist maraviroc. Moreover, qPCR analysis indicated that gp120-induced expression of MUC5AC (Fig. 2C, left panel) and $GABA_AR\alpha2$ (Fig. 2C, right panel) in NHBE was suppressed by the CXCR4 antagonist (AMD3100) but not by CCR5 antagonist (maraviroc). Thus, in NHBE cells gp120 promotes mucus formation through CXCR4.

HIV gp120 induces mucus formation via the α7-nAChR/$GABA_AR$ pathway in NHBE cells: We have demonstrated that nicotine *per se* stimulates mucus production in NHBE cells through α7-nAChRs, and the induction of mucus by IL-13 in these cells is also regulated by α7-nAChRs (Gundavarapu et al., 2012). Moreover, activation of α7-nAChR by nicotine or ACh increases the expression of $GABA_ARs$, specifically the $GABA_AR\alpha2$ subtype (Gundavarapu et al., 2012), and $GABA_ARs$ have been shown to be critical for airway mucus production (Xiang et al., 2007). However, mucus formation is also regulated by EGFR that is not affected by nAChR antagonists, indicating that EGFRs and nAChRs stimulate airway mucus formation by distinct pathways (Gundavarapu et al., unpublished observation). To ascertain whether the induction of mucus in NHBE cells by gp120 uses nAChRs and/or EGFR, prior to addition of gp120 NHBE cells were incubated with α7-nChR-specific antagonists methyllycaconitine (MLA) or the conotoxin peptide ArIB[V11L, V16D] (ArlB) (Gundavarapu et al., 2012; Whiteaker et al., 2007), or the EGFR antagonist AG1478 at predetermined optimal concentrations. As seen in Fig. 3A, while AG1478 inhibited EGF stimulated mucus in NHBE cells, it had

no detectable effect on gp120-induced mucus in these cells. On the other hand, α7-nAChR inhibitors (MLA and ArIB) suppressed the mucus formation in NHBE cells in response to gp120. Thus HIV gp120 uses the α7-nAChR pathway but not the EGFR pathway to induce mucus formation in NHBE cells. These results were further supported by qPCR analysis to determine the effects of these inhibitors on the expression of gp120-induced MUC5AC (Fig. 3B, left panel) and $GABA_AR\alpha2$ (Fig. 3B, right panel) in NHBE cells. Interestingly, in 1992 HIV-1 gp120 was shown to compete with α-bungarotoxin binding in neuronal cells, indicating binding of gp120 to nAChRs (Bracci et al., 1992); however, our studies suggest that in the mucus production by NHBE cells the effects of gp120 are mediated through binding to CXCR4, and α7-nAChRs are downstream of this event. More recently the effects of gp120-induced neuronal cell death have been linked to upregulation of nAChRs through its binding to CXCR4 (Ballester et al., 2012). Together these results suggest that CXCR4 communicate with nAChRs in affecting biological functions, including mucus production.

HIV and SIV infections induce airway mucus formation: HIV infection increases the risk of lung diseases such as COPD, asthma and bronchitis (Gingo et al., 2010; Morris et al., 2011), and mucus hypersecretion plays an important role in the pathogenesis of these diseases (Rose and Voynow, 2006; Turner and Jones, 2009). To ascertain whether HIV and SIV infections are associated with mucus production in the lungs, we examined autopsied lung tissues from control, HIV-infected, and HIV-infected + HAART patients. These lung sections were examined for mucus by alcian blue-periodic Schiff (AB-PAS) staining (Fig. 4A) and for $GABA_AR\alpha2$ expression by immunohistochemistry

(IHC) (Fig. 4B), and the results indicate that control human lungs (n = 7) have very low level of airway mucus; however, lungs from HIV-infected without HAART (n = 2) and HIV-infected + HAART (n = 6) have significantly higher levels of mucus and $GABA_AR\alpha2$. Many lung sections were from HIV-infected intravenous drug users (IVDU); however, in the absence of HIV infection, lung tissues from IVDU (n = 2) did not contain significant levels of mucus (not shown) indicating that in the absence of HIV infection, intravenous drug use was not significantly associated with excessive mucus production in the lung. Because, many HIV patients die from lung diseases, including infection, it was possible that mucus formation in HIV-infected patients might have resulted not from HIV infection *per se* but in response to a secondary bacterial/fungal infection in the lung. Therefore, we obtained lung tissues from uninfected (n = 2) and SIV-infected rhesus macaques without ART (n=3) and with ART treatment (n=4). At sacrifice the monkeys were relatively healthy and ART had decreased viremia by more than 99.5%. As in HIV-negative humans, control macaque lungs had low levels of mucus expression; however, SIV infection increased mucus production in the lung, and ART only moderately decreased the mucus (Fig. 4C). Similar results were obtained when the lungs were scored for $GABA_AR\alpha2$ by IHC (Fig. 4D) and the number of goblet cells (MCs)/mm basal lamina microscopically (Fig. 4E). Together these results suggest that both HIV and SIV infections promote mucus formation in the lung and HAART/ART does not completely block this response.

Lungs from HIV/SIV-infected and ART-treated humans and monkeys exhibit gp120 immunoreactivity: If the viral components such as gp120 were to promote

airway mucus formation, it should be present in HIV-infected lungs even after ART. To ascertain whether increased mucus in HIV/SIV-infected lungs correlated with the presence of gp120 in the lungs, we determined whether gp120 persisted in the lung sections from HIV/SIV-infected humans/monkeys even after HAART/ART. It is clear from IHC data that gp120 immunoreactivity is present in SIV-infected and SIV + ART macaque lungs (Fig. 5A), even though ART had reduced viremia by more than 99.5% in these animals (not shown). Moreover, while the gp120 immunoreactivity was widely scattered in the lungs of SIV-infected animals, it was localized mainly to macrophage-like cells in SIV + ART lungs. In addition, SIV-infected lungs had small fibrotic areas that were essentially absent in SIV + ART animals, indicating ART suppressed the SIV-induced fibrotic response in the lung. Similarly, the gp120 immunoreactivity was also seen in the lungs of HIV patients with and without HAART (Fig. 5B); however, it was difficult to determine whether HAART had any significant effect on gp120 or mucus levels in the lung. Thus a significant level of gp120 might be present in HIV/SIV-infected lungs even after HAART/ART.

The reason(s) for the presence of gp120 in the lungs after ART is unclear. It is possible that ART does not completely eliminate viral replication (North et al., 2010) from all HIV/SIV-infected lungs. Interestingly, the bronchoalveolar lavage T cells from HIV patients with lung infections secrete gp120, suggesting that the lung environment might facilitate the production and secretion of gp120 (Rubbo et al., 2011). It is also possible that some cells with integrated HIV provirus remain transcriptionally active in the lung during HAART (Hermankova et al., 2003). Nonetheless, these results suggest that gp120 may play an important role in airway mucus production in HIV-infected patients

and SIV-infected macaques, and excessive mucus production could contribute to HIV-related obstructive lung diseases. Because HIV gp120 uses the α7-nAChR/$GABA_AR$α2 pathway to trigger airway mucus formation and mucous cell hyperplasia, α7nAChR and $GABA_AR$α2 antagonists may have beneficial effects in reducing mucus formation and development of obstructive lung diseases and lung infections in HIV-infected subjects.

Materials and Methods

***Cell Cultures*:** Cells and the media were purchased from Lonza (Basel, Switzerland) and grown at air-liquid interphase (ALI) using standard procedures (Atherton et al., 2003). Alternatively, pre-differentiated NHBE cells (EpiAirway™ Tissue Model) were purchased from Mattek (Ashland, MA).

SIV infection: SIVmacR71/17E was originally prepared from pooled brain homogenates from macaques infected with R17 and R17E (Sharma et al., 1992). Virus stock was prepared from ConA-activated peripheral blood mononuclear cells depleted of CD8+ T cells, and assayed for infectivity as described (Schweighardt et al., 2004). Animals were inoculated intravenously with approximately 10^4 PFU of virus.

***Lung tissue collection from macaques*:** Animals were anesthetized with intramuscular injection of ketamine (3mg/kg) and medetomidine (0.15mg/kg) and exsanguinated from the descending aorta. Tissue samples were fixed in 10% formalin for histopathological analysis. The experimental protocol was approved by the

Institutional Animal Care and Use Committee, and the research conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

***Human Lung Tissues and Sections*:** Frozen and paraffin-embedded sections from HIV-infected, HIV-infected on HAART, and HIV-negative-IVDU were obtained from the Manhattan HIV Brain Bank (New York, NY). The IVDU in this study were mainly heroin and/or cocaine abusers (Dhillon et al., 2011). Normal (control) lung tissues were collected for emphysema studies at the Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburg.

***Mucus staining and Immunohistochemistry*:** NHBE cells at ALI were fixed in 10% neutral buffered formalin and embedded in paraffin; 5-µm-thick sections were stained for mucus with AB-PAS (El-Zimaity et al., 1998; Harkema and Hotchkiss, 1992). Briefly, after deparaffinization, the slides were stained with AB solution at pH 2.5 for 30 min, washed, and treated with 1% periodic acid for 10 min. The slides were stained with Schiff's reagent for 10 min, rinsed 3x with sodium metabisulfite, washed, dehydrated, mounted, and examined microscopically at indicated magnification. Human lung tissue sections and macaque lung tissue sections were also stained for mucus following the same procedure as mentioned above. Quantification of mucus positive cells per millimeter of basal lamina was performed using the VisioMorph system (Visiopham, Horsholm, Denmark).

Immunohistochemical staining was performed according to the protocol previously described (El-Zimaity et al., 1998). Mouse Monoclonal anti-MUC5AC (45M1; Thermo

Scientific, Lafayette, CO) and rabbit polyclonal anti-$GABA_AR\alpha2$ (Sigma, St. Louis, MO) antibodies were used at pre-optimized concentrations of 1:100 and 1:50 dilutions, respectively. The tissue sections were treated with the primary antibodies overnight at 4°C, followed by incubation with biotinylated secondary antibody (VECTASTAIN® Elite ABC kit, Vector Laboratories, Burlingame, CA). Binding was visualized using an avidin-biotinylated enzyme complex (VECTASTAIN® Elite ABC kit) with 3, 3'-diaminobenzidine (DAB) as substrate.

***Cell treatments*:** Unless indicated otherwise, NHBE cells were treated with 100 ng/ml of HIV-1 $gp120_{IIIB}$ ($gp120_{LAV}$), gp120 96ZM651 ($gp120_{ZM}$), and $gp120_{MN}$ and obtained from Dr. Madhavan Nair (Florida International University, Miami, FL). The inhibitors of nAChRs, EGFR, CXCR4, and CCR5 were purchased from Sigma (St. Louis). The inhibitors were used at the following concentrations: α7-nAChR inhibitors MLA (1 μM), CXCR4 inhibitor AMD3100 (10 μM), EGFR inhibitor AG1478 (5 μM), and CCR5 inhibitor Maraviroc (10 μM), and added to the cell cultures 2 h prior to the addition of gp120 or EGF. The conotoxin peptide inhibitor of α7-nAChRs (ArIB) was prepared as described previously (Ellison et al., 2006) and used at 500 nM. (Gundavarapu et al., 2012).

***RT-PCR and qPCR*:** Total RNA from human and macaque lungs and NHBE cells was isolated using TRI-Reagent (Molecular Research Center, Inc., Cincinnati, OH). qPCR was performed using Step One plus Detection System (Applied Biosystems, Foster City, CA) and TaqMan One-Step RT-PCR kit containing AmpliTaq Gold® DNA polymerase. Specific primers and probes for MUC5AC and $GABA_AR\alpha2$ were obtained

from Applied Biosystems. Fold changes in qPCR expression were calculated by the $2^{(-\Delta\Delta CT)}$ method (Livak and Schmittgen, 2001).

***Statistical analysis*:** All data were analyzed using Graph-Pad Prism software 5.03 (Graph-Pad Software Inc., San Diego, CA). One-way ANOVA was used to compare mean between the groups using Tukey post-hoc test that compares all groups at 95% confidence intervals. Results are presented as the means $\pm$ SD. The differences with p value of ≤ 0.05 were considered statistically significant. *P ≤ 0.05; **P ≤ 0.01, ***P ≤ 0.001.

Acknowledgements

The authors would like to thank Thomas Gagliano for help with the figures. Supported in part by grants from the US Army Medical Research and Material Command (GW093005), Lovelace Respiratory Research Institute (IMMSPT) and GM103801 and GM48677.

References

Atherton, H.C., G. Jones, and H. Danahay. 2003. IL-13-induced changes in the goblet cell density of human bronchial epithelial cell cultures: MAP kinase and phosphatidylinositol 3-kinase regulation. *American journal of physiology* 285:L730-739.

Ballester, L.Y., C.M. Capo-Velez, W.F. Garcia-Beltran, F.M. Ramos, E. Vazquez-Rosa, R. Rios, J.R. Mercado, R.I. Melendez, and J.A. Lasalde-Dominicci. 2012. Up-regulation of the neuronal nicotinic receptor alpha7 by HIV glycoprotein 120: potential implications for HIV-associated neurocognitive disorder. *The Journal of biological chemistry* 287:3079-3086.

Bracci, L., L. Lozzi, M. Rustici, and P. Neri. 1992. Binding of HIV-1 gp120 to the nicotinic receptor. *FEBS letters* 311:115-118.

Crothers, K., A.A. Butt, C.L. Gibert, M.C. Rodriguez-Barradas, S. Crystal, A.C. Justice, and T. Veterans Aging Cohort 5 Project. 2006. Increased COPD among HIV-positive compared to HIV-negative veterans. *Chest* 130:1326-1333.

Dhillon, N.K., F. Li, B. Xue, O. Tawfik, S. Morgello, S. Buch, and A.O. Ladner. 2011. Effect of cocaine on human immunodeficiency virus-mediated pulmonary endothelial and smooth muscle dysfunction. *American journal of respiratory cell and molecular biology* 45:40-52.

Diaz, P.T., M.A. King, E.R. Pacht, M.D. Wewers, J.E. Gadek, H.N. Nagaraja, J. Drake, and T.L. Clanton. 2000. Increased susceptibility to pulmonary emphysema among HIV-seropositive smokers. *Annals of internal medicine* 132:369-372.

Diaz, P.T., M.D. Wewers, E. Pacht, J. Drake, H.N. Nagaraja, and T.L. Clanton. 2003. Respiratory symptoms among HIV-seropositive individuals. *Chest* 123:1977-1982.

Eddleston, J., S.C. Christiansen, and B.L. Zuraw. 2002. Functional expression of the C-X-C chemokine receptor CXCR4 by human bronchial epithelial cells: regulation by proinflammatory mediators. *J Immunol* 169:6445-6451.

El-Zimaity, H.M., H. Ota, S. Scott, D.E. Killen, and D.Y. Graham. 1998. A new triple stain for Helicobacter pylori suitable for the autostainer: carbol fuchsin/Alcian blue/hematoxylin-eosin. *Archives of pathology & laboratory medicine* 122:732-736.

Ellison, M., C. Haberlandt, M.E. Gomez-Casati, M. Watkins, A.B. Elgoyhen, J.M. McIntosh, and B.M. Olivera. 2006. Alpha-RgIA: a novel conotoxin that specifically and potently blocks the alpha9alpha10 nAChR. *Biochemistry* 45:1511-1517.

Forey, B.A., A.J. Thornton, and P.N. Lee. 2011. Systematic review with meta-analysis of the epidemiological evidence relating smoking to COPD, chronic bronchitis and emphysema. *BMC Pulm Med* 11:36.

Fu, X.W., K. Wood, and E.R. Spindel. 2011. Prenatal nicotine exposure increases GABA signaling and mucin expression in airway epithelium. *American journal of respiratory cell and molecular biology* 44:222-229.

Gingo, M.R., M.P. George, C.J. Kessinger, L. Lucht, B. Rissler, R. Weinman, W.A. Slivka, D.K. McMahon, S.E. Wenzel, F.C. Sciurba, and A. Morris. 2010. Pulmonary function abnormalities in HIV-infected patients during the current antiretroviral therapy era. *Am J Respir Crit Care Med* 182:790-796.

Gonzalo, J.A., C.M. Lloyd, A. Peled, T. Delaney, A.J. Coyle, and J.C. Gutierrez-Ramos. 2000. Critical involvement of the chemotactic axis CXCR4/stromal cell-derived factor-1 alpha in the inflammatory component of allergic airway disease. *J Immunol* 165:499-508.

Grubb, J.R., A.C. Moorman, R.K. Baker, and H. Masur. 2006. The changing spectrum of pulmonary disease in patients with HIV infection on antiretroviral therapy. *Aids* 20:1095-1107.

Gundavarapu, S., J.A. Wilder, N.C. Mishra, J. Rir-Sima-Ah, R.J. Langley, S.P. Singh, A.I. Saeed, R.J. Jaramillo, K.M. Gott, J.C. Pena-Philippides, K.S. Harrod, J.M. McIntosh, S. Buch, and M.L. Sopori. 2012. Role of nicotinic receptors and acetylcholine in mucous

cell metaplasia, hyperplasia, and airway mucus formation in vitro and in vivo. *The Journal of allergy and clinical immunology* 130:770-780 e711.
Harkema, J.R., and J.A. Hotchkiss. 1992. In vivo effects of endotoxin on intraepithelial mucosubstances in rat pulmonary airways. Quantitative histochemistry. *The American journal of pathology* 141:307-317.
Hermankova, M., J.D. Siliciano, Y. Zhou, D. Monie, K. Chadwick, J.B. Margolick, T.C. Quinn, and R.F. Siliciano. 2003. Analysis of human immunodeficiency virus type 1 gene expression in latently infected resting CD4+ T lymphocytes in vivo. *Journal of virology* 77:7383-7392.
Hovenberg, H.W., J.R. Davies, and I. Carlstedt. 1996. Different mucins are produced by the surface epithelium and the submucosa in human trachea: identification of MUC5AC as a major mucin from the goblet cells. *Biochem J* 318 (Pt 1):319-324.
Kanmogne, G.D., C. Primeaux, and P. Grammas. 2005. HIV-1 gp120 proteins alter tight junction protein expression and brain endothelial cell permeability: implications for the pathogenesis of HIV-associated dementia. *J Neuropathol Exp Neurol* 64:498-505.
Kaul, M., G.A. Garden, and S.A. Lipton. 2001. Pathways to neuronal injury and apoptosis in HIV-associated dementia. *Nature* 410:988-994.
Livak, K.J., and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif* 25:402-408.
Morris, A., M.P. George, K. Crothers, L. Huang, L. Lucht, C. Kessinger, and E.C. Kleerup. 2011. HIV and chronic obstructive pulmonary disease: is it worse and why? *Proc Am Thorac Soc* 8:320-325.
Murray, J.F., and J. Mills. 1990. Pulmonary infectious complications of human immunodeficiency virus infection. Part II. *Am Rev Respir Dis* 141:1582-1598.
Niaura, R., W.G. Shadel, K. Morrow, K. Tashima, T. Flanigan, and D.B. Abrams. 2000. Human immunodeficiency virus infection, AIDS, and smoking cessation: the time is now. *Clin Infect Dis* 31:808-812.
North, T.W., J. Higgins, J.D. Deere, T.L. Hayes, A. Villalobos, L. Adamson, B.L. Shacklett, R.F. Schinazi, and P.A. Luciw. 2010. Viral sanctuaries during highly active antiretroviral therapy in a nonhuman primate model for AIDS. *Journal of virology* 84:2913-2922.
Palella, F.J., Jr., K.M. Delaney, A.C. Moorman, M.O. Loveless, J. Fuhrer, G.A. Satten, D.J. Aschman, and S.D. Holmberg. 1998. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. *N Engl J Med* 338:853-860.
Raynaud, C., N. Roche, and C. Chouaid. 2011. Interactions between HIV infection and chronic obstructive pulmonary disease: Clinical and epidemiological aspects. *Respir Res* 12:117.
Rose, M.C., and J.A. Voynow. 2006. Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiological reviews* 86:245-278.
Rubbo, P.A., E. Tuaillon, K. Bollore, V. Foulongne, A. Bourdin, N. Nagot, P. Van de Perre, C. Desgranges, D. Israel-Biet, and J.P. Vendrell. 2011. The potential impact of CD4+ T cell activation and enhanced Th1/Th2 cytokine ratio on HIV-1 secretion in the lungs of individuals with advanced AIDS and active pulmonary infection. *Clinical immunology* 139:142-154.
Rychert, J., D. Strick, S. Bazner, J. Robinson, and E. Rosenberg. 2010. Detection of HIV gp120 in plasma during early HIV infection is associated with increased proinflammatory and immunoregulatory cytokines. *AIDS Res Hum Retroviruses* 26:1139-1145.
Santosuosso, M., E. Righi, V. Lindstrom, P.R. Leblanc, and M.C. Poznansky. 2009. HIV-1 envelope protein gp120 is present at high concentrations in secondary lymphoid organs of individuals with chronic HIV-1 infection. *J Infect Dis* 200:1050-1053.

Schweighardt, B., A.M. Roy, D.A. Meiklejohn, E.J. Grace, 2nd, W.J. Moretto, J.J. Heymann, and D.F. Nixon. 2004. R5 human immunodeficiency virus type 1 (HIV-1) replicates more efficiently in primary CD4+ T-cell cultures than X4 HIV-1. *Journal of virology* 78:9164-9173.

Segal, L.N., B.A. Methe, A. Nolan, Y. Hoshino, W.N. Rom, R. Dawson, E. Bateman, and M.D. Weiden. 2011. HIV-1 and bacterial pneumonia in the era of antiretroviral therapy. *Proc Am Thorac Soc* 8:282-287.

Sharma, D.P., M.C. Zink, M. Anderson, R. Adams, J.E. Clements, S.V. Joag, and O. Narayan. 1992. Derivation of neurotropic simian immunodeficiency virus from exclusively lymphocytetropic parental virus: pathogenesis of infection in macaques. *Journal of virology* 66:3550-3556.

Turner, J., and C.E. Jones. 2009. Regulation of mucin expression in respiratory diseases. *Biochem Soc Trans* 37:877-881.

Voynow, J.A., and B.K. Rubin. 2009. Mucins, mucus, and sputum. *Chest* 135:505-512.

Whiteaker, P., S. Christensen, D. Yoshikami, C. Dowell, M. Watkins, J. Gulyas, J. Rivier, B.M. Olivera, and J.M. McIntosh. 2007. Discovery, synthesis, and structure activity of a highly selective alpha7 nicotinic acetylcholine receptor antagonist. *Biochemistry* 46:6628-6638.

Wilen, C.B., J.C. Tilton, and R.W. Doms. 2012. HIV: cell binding and entry. *Cold Spring Harbor perspectives in medicine* 2:

Xiang, Y.Y., S. Wang, M. Liu, J.A. Hirota, J. Li, W. Ju, Y. Fan, M.M. Kelly, B. Ye, B. Orser, P.M. O'Byrne, M.D. Inman, X. Yang, and W.Y. Lu. 2007. A GABAergic system in airway epithelium is essential for mucus overproduction in asthma. *Nat Med* 13:862-867.

FIGURE LEGENDS

Figure 1: HIV gp120 induces mucus formation and increases MUC5AC and $GABA_AR\alpha2$ expression in NHBE cells. (A): NHBE cells were treated with 10, 33, and 100 ng/ml of $gp120_{LAV}$ for 48 hr and the mucus in the inserts was visualized by AB-PAS staining. **(B):** NHBE cells were also treated with 100 ng/ml of $gp120_{LAV}$, $gp120_{MN}$, or $gp120_{ZM}$ and examined for mucus formation by AB-PAS staining. **(C):** The expression of MUC5AC and $GABA_AR\alpha2$ by qPCR. Each experiment was repeated at least three times. Error bars on MUC5AC and $GABA_AR\alpha2$ qPCR analysis represent mean ± SEM from three inserts.

Figure 2: NHBE cells express CXCR4 but not CCR5 and HIV gp120 induces mucus through CXCR4. (A): NHBE cell RNA was analyzed by qPCR for the expression of CXCR4 and CCR5 up to 40 cycles of amplification. **(B):** $gp120_{LAV}$-induced mucus formation was determined in the presence or absence of CXCR4 inhibitor AMD3100 and CCR5 inhibitor maraviroc . **(C)**: gp120-induced mRNA expression of MUC5AC and $GABA_AR\alpha2$ was determined in the presence and absence of AMD3100 and maraviroc by qPCR analysis. Each experiment was repeated at least three times; bars represent mean ± SEM.

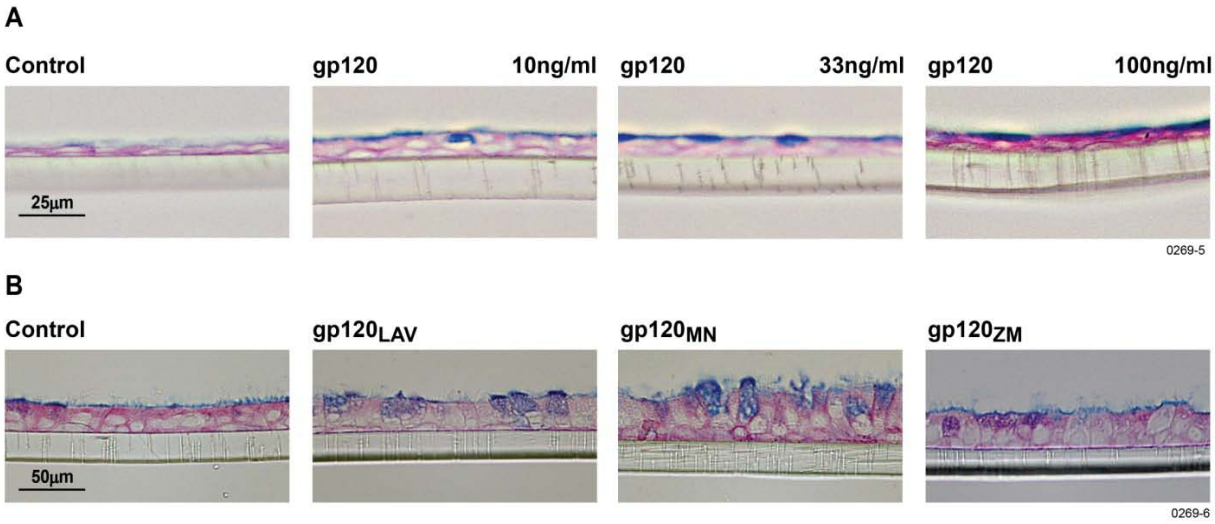
Figure 3: HIV gp120 induces mucus formation through α7-nAChRs in NHBE cells. **(A):** NHBE cells were cultured with EGF or $gp120_{LAV}$ as described in Materials and Methods. Where indicated, EGFR inhibitor (AG1478) or α7-nAChR inhibitors (MLA or ArIB) were added 2 h before the addition of gp120/EGF. Cells were stained for mucus

(AB-PAS). **(B):** Expression of MUC5AC and $GABA_AR\alpha2$ mRNA was determined by qPCR analysis. The results represent three independent experiments and error bars are mean ± SEM from three inserts.

Figure 4: HIV and SIV infections increase mucus and $GABA_AR\alpha2$ expression in the lung. Lung sections from control, HIV-infected, and HIV + HAART patients were **(A):** stained for AB-PAS for mucus and **(B):** stained using IHC for $GABA_AR\alpha2$. Similarly, lung sections from control, SIV-infected, and SIV + ART monkeys were **(C):** stained AB-PAS and **(D):** IHC stained for $GABA_AR\alpha2$. **(E):** Mucus containing cells (MC's)/mm basal lamina was enumerated microscopically in lung sections from control, SIV-infected, and SIV + ART lung sections. Error bars in **(E)** represent data from at least 5 different lung sections from each group.

Figure 5: SIV- and HIV-infected lungs exhibit gp120 immunoreactivity even after ART. Lungs sections from control, SIV, and SIV + ART **(A),** and control, HIV-infected, and HIV + HAART **(B)** were stained for gp120 immunoreactivity as described in Materials and Methods.

FIGURE 1


A
Control
gp120 10ng/ml
gp120 33ng/ml
gp120 100ng/ml
25µm
0269-5
B
Control
gp120LAV
gp120MN
gp120ZM
50µm
0269-6

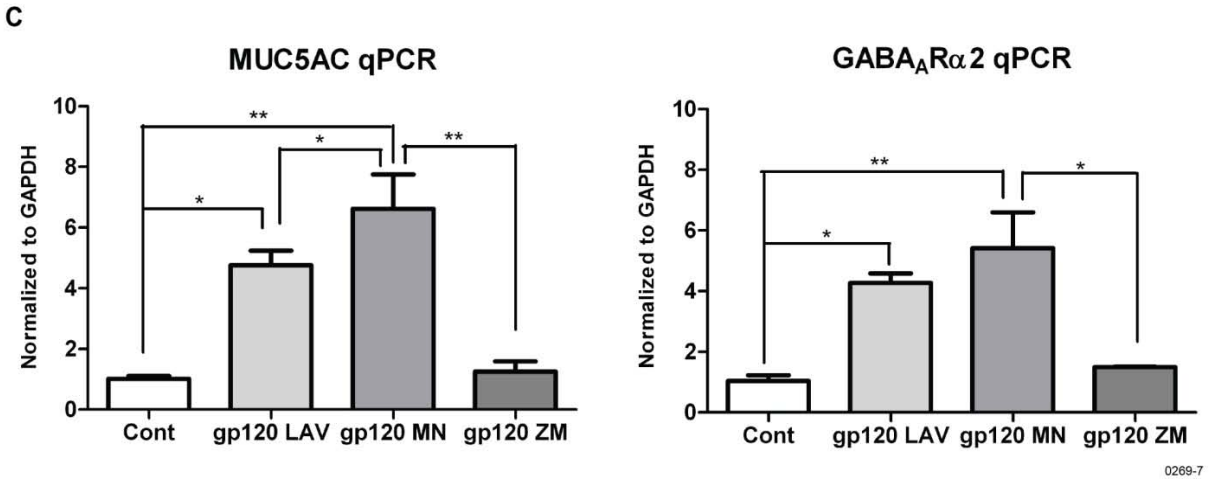

C
MUC5AC qPCR
GABAARα2 qPCR
Normalized to GAPDH
Cont
gp120 LAV
gp120 MN
gp120 ZM
0269-7

FIGURE 2

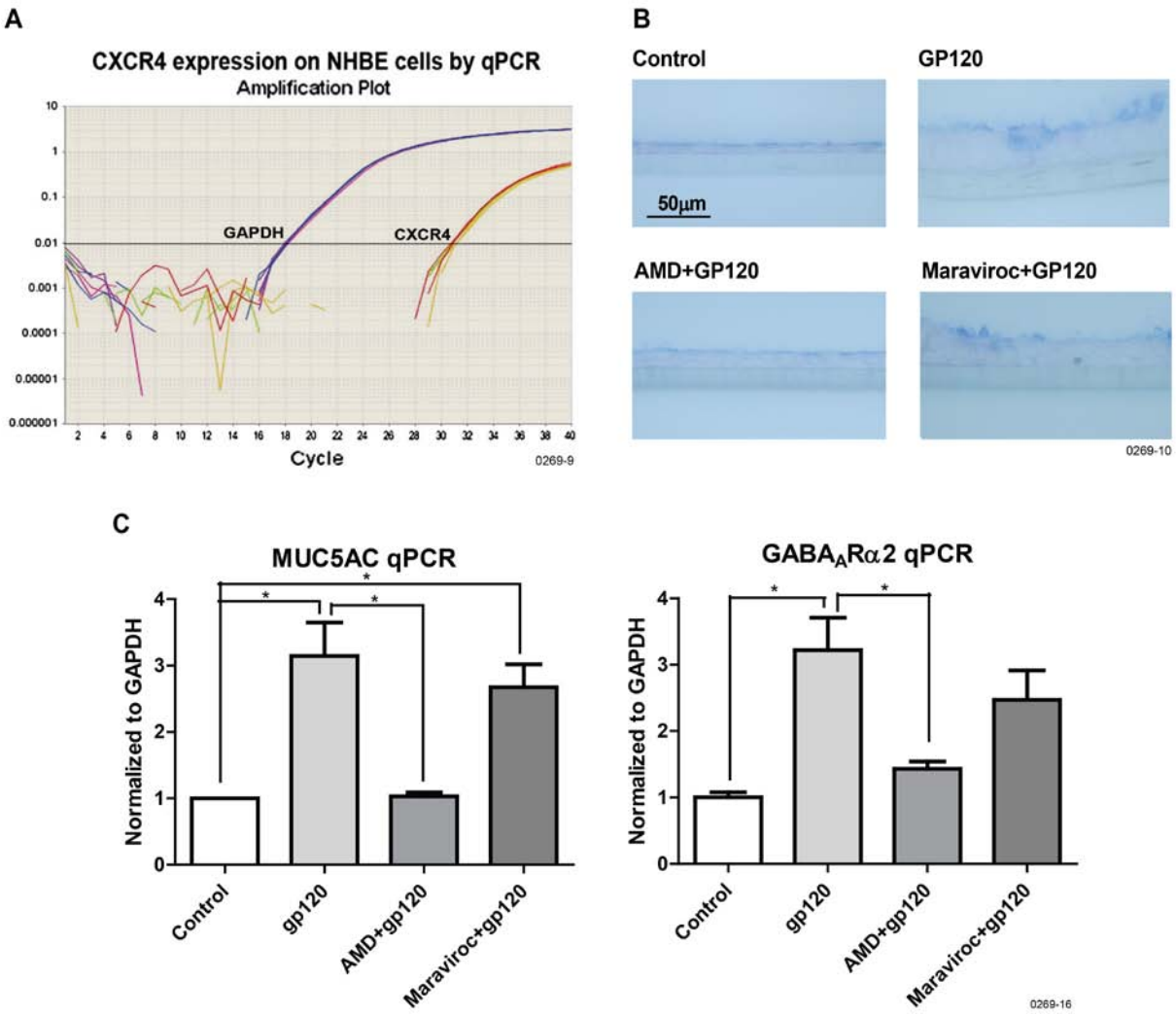
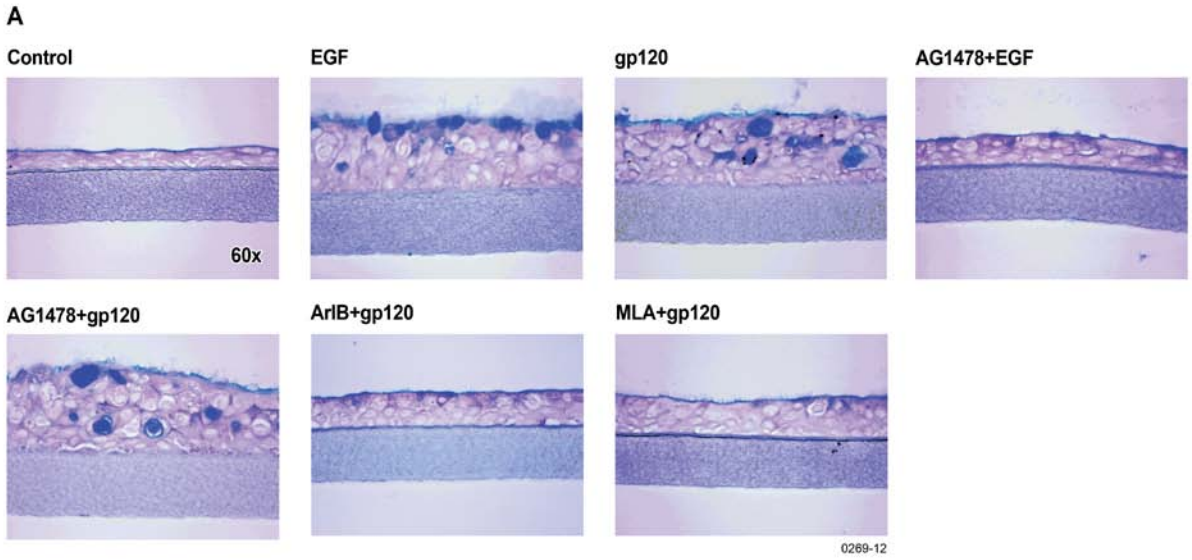

A
CXCR4 expression on NHBE cells by qPCR
Amplification Plot
GAPDH
CXCR4
Cycle
0269-9
B
Control
GP120
50µm
AMD+GP120
Maraviroc+GP120
0269-10
C
MUC5AC qPCR
Normalized to GAPDH
Control
gp120
AMD+gp120
Maraviroc+gp120
GABA$_A$Rα2 qPCR
Normalized to GAPDH
Control
gp120
AMD+gp120
Maraviroc+gp120
0269-16

FIGURE 3


A
Control
EGF
gp120
AG1478+EGF
60x
AG1478+gp120
ArlB+gp120
MLA+gp120
0269-12

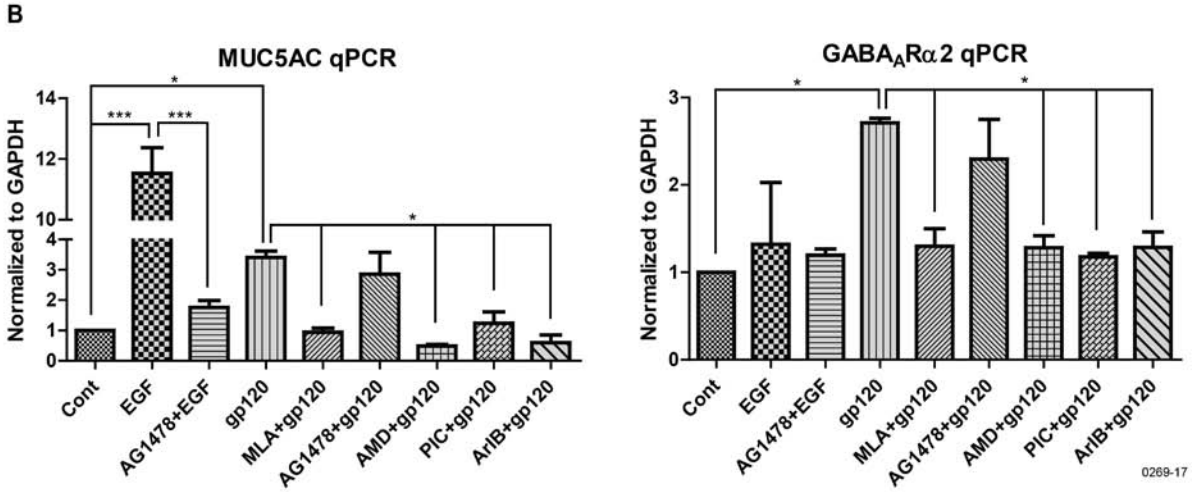
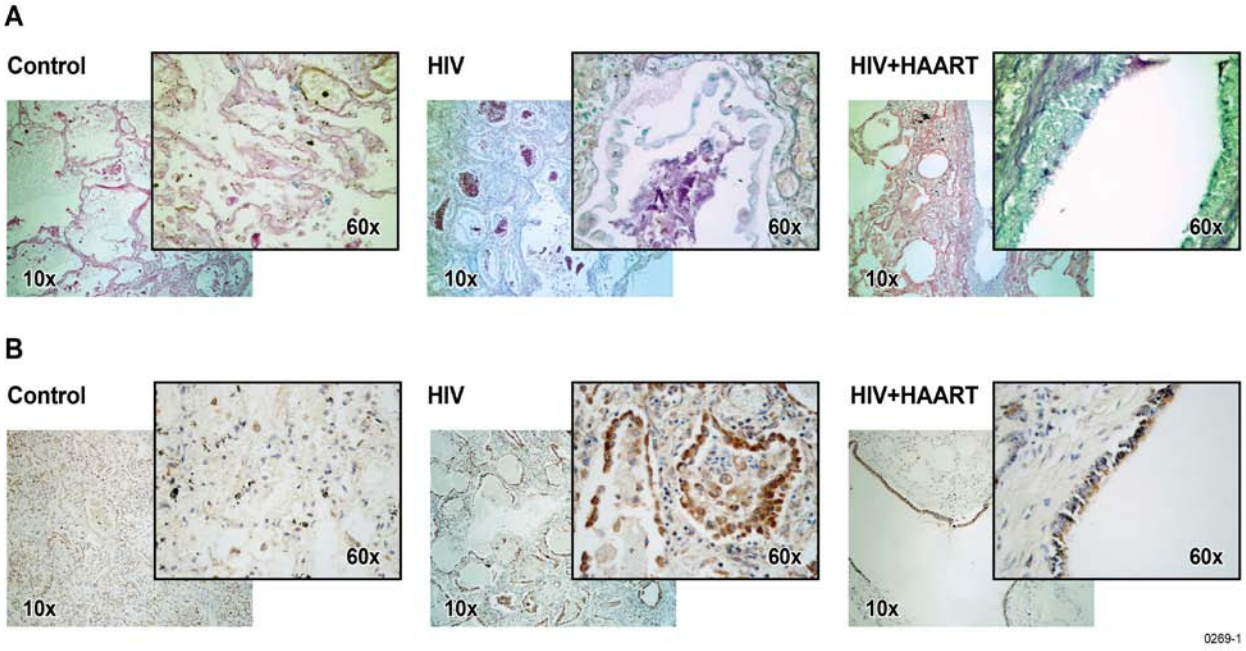
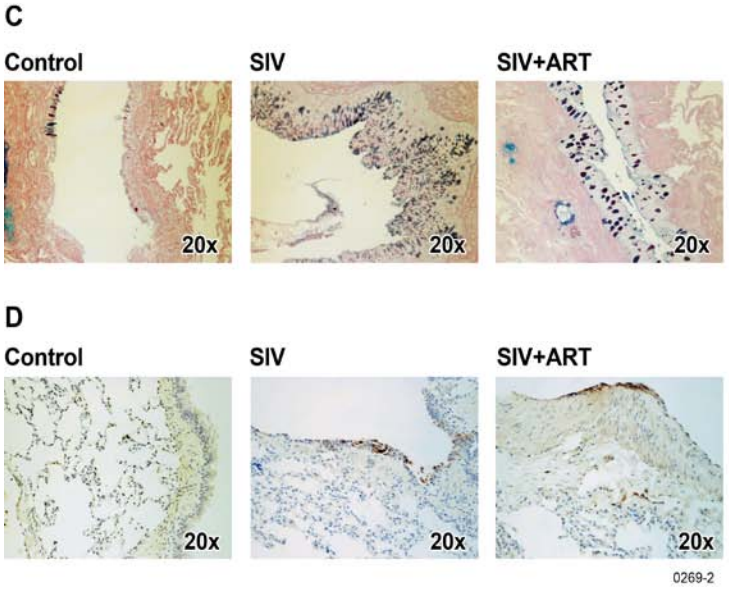

B
MUC5AC qPCR
Normalized to GAPDH
14
12
10
4
3
2
1
0
***
***
*
*
Cont
EGF
AG1478+EGF
gp120
MLA+gp120
AG1478+gp120
AMD+gp120
PIC+gp120
ArlB+gp120
GABA$_A$Rα2 qPCR
Normalized to GAPDH
3
2
1
0
*
*
Cont
EGF
AG1478+EGF
gp120
MLA+gp120
AG1478+gp120
AMD+gp120
PIC+gp120
ArlB+gp120
0269-17

FIGURE 4


A
Control
HIV
HIV+HAART
60x
10x
B
Control
HIV
HIV+HAART
60x
10x
0269-1


C
Control
SIV
SIV+ART
20x
D
Control
SIV
SIV+ART
20x
0269-2

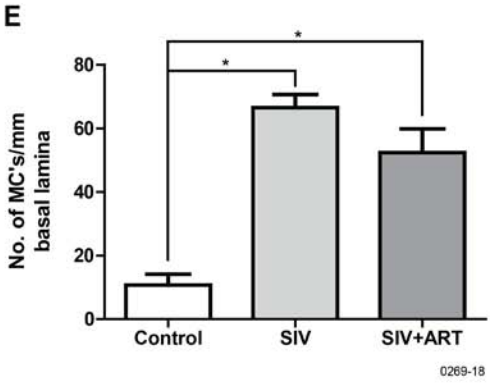
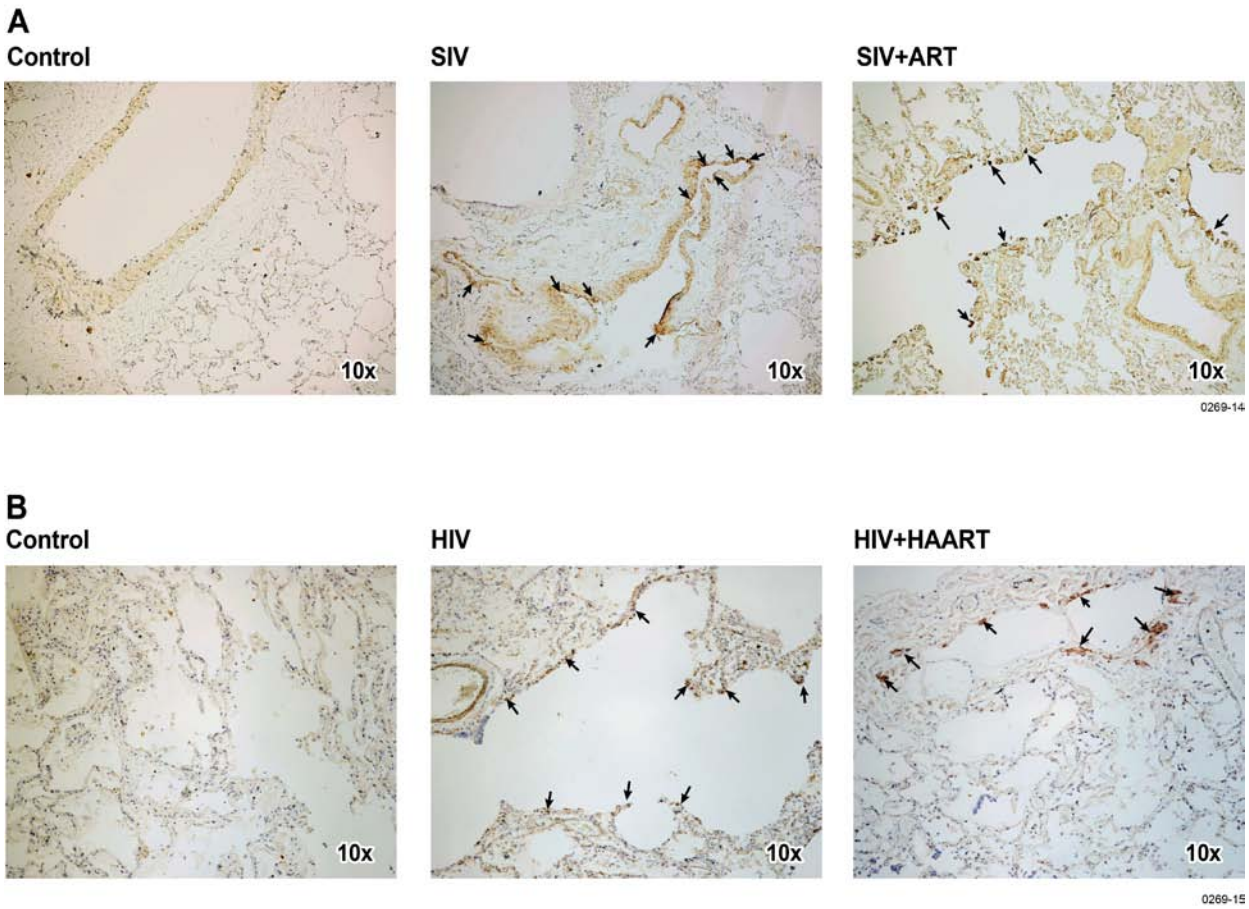

E
No. of MC's/mm basal lamina
80
60
40
20
0
*
*
Control
SIV
SIV+ART
0269-18

FIGURE 5


A
Control
SIV
SIV+ART
10x
10x
10x
0269-14
B
Control
HIV
HIV+HAART
10x
10x
10x
0269-15